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Oxidative stress-induced expression of haem oxygenase-1 (HO-1) in human mononuclear cells in vivo and ex vivo and the impact of lycopene supplementation

Markovitch, Daniella

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**OXIDATIVE STRESS-INDUCED EXPRESSION OF HAEM OXYGENASE-1
(HO-1) IN HUMAN MONONUCLEAR CELLS *IN VIVO* AND *EX VIVO* AND
THE IMPACT OF LYCOPENE SUPPLEMENTATION**

Daniella Markovitch

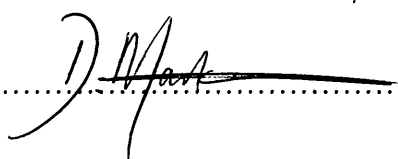
A thesis submitted for the degree of Doctor of Philosophy
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May 2005

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ABSTRACT

Oxidative stress is a feature of many chronic diseases. Two approaches proposed to reduce the risk of disease include regular exercise and consumption of a diet rich in fruit and vegetables, as both behaviours can improve the antioxidant defence system and thereby attenuate oxidative stress. In particular, recent evidence has demonstrated a reduced risk of certain diseases with increased consumption of tomato products, in which the carotenoid lycopene is the principal antioxidant. The research reported in this thesis was conducted in two stages; firstly, two models of oxidative stress were characterised prior to progression on to stage two, where the research aimed to investigate the impact of lycopene supplementation on modulation of haem oxygenase-1 (HO-1) expression in response to oxidative stress.

Expression of the oxidant-responsive enzyme HO-1 is the most appropriate candidate gene to assess oxidative stress *in vivo*. In the first model of oxidative stress employed in the present thesis, both prolonged and intermittent running increased HO-1 mRNA expression in human mononuclear cells, although considerable inter-individual variation in the HO-1 response to exercise was evident. A second model was therefore developed to further explore the cellular response to oxidative stress using hydrogen peroxide (H₂O₂) treatment of freshly harvested lymphocytes and monocytes *ex vivo*. The HO-1 protein response to H₂O₂ was a reproducible response and was used as a subsequent stressor following an acute bout of exercise. In this investigation, increased lymphocyte HO-1 mRNA 2 h post-exercise was associated with a reduced induction of HO-1 protein in the *ex vivo* model, possibly indicating an adaptive response to prior exercise.

The second part of the research reported in this thesis demonstrates that tomato supplementation significantly increased plasma lycopene levels. However, the basal and induced level of HO-1 protein was not altered with supplementation. Furthermore, cell viability following H₂O₂ treatment did not differ with supplementation although the basal level of apoptosis was significantly lower in lymphocytes following tomato supplementation. As the basal and induced level of HO-1 protein was not modulated by tomato supplementation, it would appear that the reduction in lymphocyte apoptosis is mediated by some non-oxidative mechanism of lycopene. The objectively determined physical activity level of the subjects used in this investigation was high and it is possible this may have affected the response to the intervention. As 60% of the global population is inactive, it is possible that future work should examine the effect of tomato supplementation in sedentary populations that have not benefited from the adaptations to regular physical activity.

PUBLICATIONS

Conference Proceedings

Markovitch, D., Thompson, D. and Tyrrell, R.M. (2004). Haem oxygenase-1 induction *ex vivo* following hydrogen peroxide treatment of human lymphocytes and monocytes. *J. Physiol.* **555P** PC74

Markovitch, D., Thompson, D. and Tyrrell, R.M. (2004). Reproducibility of haem oxygenase-1 protein induction following hydrogen peroxide treatment in human lymphocytes and monocytes. *J. Physiol.* **565P** PC20

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ABBREVIATIONS

AAPH	2, 2'-Azobis-(2-amidinopropane)-dihydrochloride
ACSM	American College of Sports Medicine
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
AP-1	Activator protein-1
Apaf-1	Apoptosis-inducing factor-1
ATP	Adenosine 5'-triphosphate
AV ⁺	Annexin V positive
BHT	Butylated hydroxytoluene
BMR	Basal metabolic rate
BR	Bilirubin
BSA	Bovine serum albumin
Ca ²⁺	Calcium
CAT	Catalase
CDC	Centers for Disease Control and Prevention
CHO	Carbohydrate
CO	Carbon monoxide
CoA	Coenzyme A
CoQ	Coenzyme Q
CVD	Cardiovascular disease
DLW	Doubly labelled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ES	Effect size
ESR	Electron spin resonance
FACS	Fluorescence Activated Cell Sorting
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
FITC	Fluorescein isothiocyanate
FL1	Fluorescent channel 1
FL2	Fluorescent channel 2
FL3	Fluorescent channel 3

GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione disulphide (oxidised)
Hb	Haemoglobin
HBO	Hyperbaric oxygen
HDL	High-density lipoprotein
HIE	High-intensity exercise
HNE	4-hydroxy-2-trans-nonenal
HO	Haem Oxygenase
HO-1	Haem Oxygenase-1
HO-2	Haem Oxygenase-2
HO-3	Haem Oxygenase-3
HOCl	Hydrogen chloride
HPLC	High performance liquid chromatography
HR	Heart rate
HR _{max}	Maximal heart rate
HRP	Horseradish peroxidase
HS	Horse serum
HSP	Heat shock protein
HX	Hypoxanthine
H ₂ O ₂	Hydrogen peroxide
IEP	Intermittent exercise protocol
IGF-I	Insulin-like growth factor-I
IL	Interleukin
IMP	Inosine 5'-monophosphate
iNOS	Inducible nitric oxide synthase
IOM	Institute of Medicine
I-R	Ischaemia-reperfusion
IU	International unit
kcal	Kilocalorie
LDL	Low-density lipoprotein
LIE	Low-intensity exercise
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
Mb	Myoglobin
MDA	Malondialdehyde
METS	Metabolic equivalents

MFI	Median fluorescence intensity
MNC	Mononuclear cells
MTP	Mitochondrial transmembrane potential
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NADH	Nicotine adenine dinucleotide (reduced)
NADPH	Nicotine adenine dinucleotide phosphate (reduced)
NF- κ B	Nuclear factor- κ B
nNOS	Neuronal nitric oxide synthase
NO $^{\bullet}$	Nitric oxide
NO $_2^{\bullet}$	Nitric dioxide
NOS	Nitric oxide synthase
O $_2$	Oxygen
O $_3$	Ozone
1 O $_2$	Singlet oxygen
O $_2^{\bullet -}$	Superoxide radical
OH $^{\bullet}$	Hydroxyl radical
ONOO $^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
8-oxoG	8-oxoguananine
8-oxodG	8-deoxyguanosine
PAL	Physical activity level
PBN	Phenyl- <i>tert</i> -butyl nitron
PBS	Phosphate buffered saline
PE	R-Phycoerythrin
PI $^+$	Propidium iodide positive
PS	Phosphatidylserine
PSA	Prostate specific antigen
PTP	Permeability transition pore
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
RO $^{\bullet}$	Alkoxyl radical
ROO $^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Media
SDS	Sodium dodecyl sulphate
SEM	Standard error of the measurement
SFM	Serum free media
SOD	Superoxide dismutase

TBARS	Thiobarbituric reactive substances
TC	Total cholesterol
TEE	Total energy expenditure
TNF- α	Tumour necrosis factor- α
UA	Uric acid
UVA	Ultraviolet A
VLDL	Very-low-density lipoprotein
$\dot{V}CO_2$	Carbon dioxide uptake
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2max}$	Maximum oxygen uptake
WBC	White blood cells
WHO	World Health Organisation
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
%CV	% coefficient of variation

CHAPTER 1

INTRODUCTION

1.1 Exercise and physical activity

The terms ‘exercise’ and ‘physical activity’ may be understood differently by different people (Booth, 2000) and, therefore, a distinction between physical activity and exercise should be made. Physical activity is defined as ‘bodily movement, produced by the contraction of muscle, substantially increasing energy expenditure’ (DHHS, 1996), whereas exercise is the ‘planned structured and repetitive bodily movement done to promote or maintain components of physical fitness’ (IOM, 2002). In the present thesis, these terms will generally be used interchangeably as many authors use both exercise and physical activity without distinguishing any differences.

1.1.1 Relationship between regular exercise and disease

Physical activity beneficially affects the body in a multi-factorial manner and is suggested to be responsible for the prevention of many chronic diseases (Booth *et al.*, 2000; Booth *et al.*, 2002). A wealth of evidence has revealed that regular physical activity is related to the reduced incidence of many chronic conditions such as cardiovascular disease (CVD), obesity, type 2 diabetes, certain cancers, and osteoporosis (McCarter, 2000; DoH, 2004). Worldwide, physical inactivity is estimated to cause approximately 1.9 million deaths annually, accounting for 23% of all cases of CVD, 16-17% of colon cancer, 15% of diabetes, 12-13% of strokes and 11% of breast cancer (WHO, 2002). In the UK, the prevalence of inactivity is increasing with two separate surveys reporting approximately two thirds of men and three quarters of women aged over 16 years participate in less than 30 min of moderate intensity activity on five or more days of the week (Stamatakis, 2003; Office for National Statistics, 2004). Furthermore, the Global Burden of Disease Study puts physical inactivity among the top ten risk factors that threaten global health (Murray and Lopez, 1996). People who are physically active reduce the risk of developing CVD, stroke and type 2 diabetes by up to 50% and the risk of premature death by 20-30% (DoH, 2004). It is recognised that regular physical activity is an important component of lifestyle and this has resulted in the development of exercise guidelines that aim to reduce the prevalence of chronic disease. However, there are numerous different public health recommendations and the

minimum frequency, duration and intensity of exercise required for good health still remains unclear (Blair *et al.*, 2004).

1.1.2 Exercise Recommendations

In the mid 1990s, reports on physical activity and health recommendations were established by the US Centers for Disease Control and Prevention (CDC) and the American College of Sports Medicine (ACSM) which were supported by the US Surgeon General's Report (1996). It was recommended that 'every US adult should accumulate 30 min or more of moderate-intensity physical activity on most, preferably all days of the week...' (DHHS, 1996). Individuals already meeting the basic recommendations were advised they could gain additional health benefits by doing more exercise, perhaps including higher intensity activities (DHHS, 1996). These guidelines were directed at reducing the public health burden of disease and therefore it was necessary that the prescribed amount of physical activity was seen as achievable. More recently, the Institute of Medicine (IOM) suggested that '...to prevent weight gain, as well as to accrue additional, weight-independent health benefits of physical activity, 60 min of moderate intensity physical activity is recommended....' (IOM, 2002). The current exercise recommendation of 30 min of moderate exercise a day is not met by at least 60% of the global population (WHO, 2002).

The health benefits gained from regular exercise participation have been classified by level of physical activity (Table 1.1). From this, it has been suggested that a level of light activity is better than inactivity, although progression to levels three and four provides superior protection against chronic diseases. The different levels of activity have been classified using metabolic equivalents (METs). This is a unit expressed as multiples of an individual's resting oxygen (O_2) uptake, with one MET defined as a rate of O_2 consumption of $3.5 \text{ ml.kg}^{-1}\text{min}^{-1}$, equivalent to the resting metabolic rate (RMR) obtained whilst sitting. The joint CDC-ACSM position statement identified three different intensity categories for physical activity (light: < 3 METs, moderate: 3-6 METs, vigorous: > 6 METs) (Pate *et al.*, 1995). In addition, a reference database of activities deemed to be of low, moderate, and vigorous intensity according to the energy expended in METs has been developed, generating a compendium of physical activities (Ainsworth *et al.*, 2000).

Table 1.1 Levels of physical activity according to recommendations

Level	Descriptor	METS	Typical activity pattern	Health benefits
1	Inactive	1	Drives/ Public transport Sedentary job Minimal household activities No active recreation	Nil
2	Lightly active	< 3	May walk/cycle Walking, lifting, carrying as part of work Some light intensity recreation	A stepping stone to the recommended level
3	Moderately active	3-6	Regular active commuting Regular work-related physical tasks Regular moderately active recreation	High level of protection
4	Very active	> 6	Regular active commuting Very active job Vigorous intensity sport	Maximal protection against chronic disease Slight increase in risk of injury
5	Highly active	> 6	Performs high volumes of vigorous or very vigorous fitness training, often in order to play sport	Maximal protection against chronic disease Increased risk of injury

METS have been added as a guide to show the relative intensity of activity performed at each descriptor, and are not intended to indicate that all activities at each level will be between these ranges. (Adapted from 'At least five a week' Department of Health, The Chief Medical Officer, 2004).

The doubly labelled water method (DLW) is generally held to be the criterion standard for estimating energy expenditure (Schoeller, 1999; Ainslie *et al.*, 2003). This method is relatively non-invasive and allows quantification of total energy expenditure over a prolonged period of time. As a result, a large number of studies have investigated physical activity using this technique and this has led to the development of a database enabling comparisons between individuals of different age groups and body mass to be made (IOM, 2002).

A limitation of the doubly labelled water method is that it only estimates total energy expenditure. Once an estimate of resting energy has been made it is possible to estimate energy expenditure above rest although it is impossible to quantify the intensity, duration or frequency of physical activity (Ainslie *et al.*, 2003). Measurement of these subcomponents of physical activity energy expenditure will enable investigators to establish whether individuals are meeting exercise recommendations and therefore improve understanding of the relationship between physical activity and health. Motion sensors have been developed and provide a crude index of physical activity intensity although this too has its limitations (Ainslie *et al.*, 2003). A new device has been designed to assess physical activity in populations and uses a combined motion sensor and heart rate monitor to predict energy expenditure from physical activity (Brage *et al.*, 2005). This has been validated and has been shown to be a reliable method of assessing physical activity (Brage *et al.*, 2005). Consequently, this type of device may improve the understanding of the dose-response relationship between physical activity and health (Ainslie *et al.*, 2003).

1.1.3 Adaptations to regular exercise

Physical inactivity potentiates at least 20 unhealthy conditions (Booth *et al.*, 2002). Appropriately performed physical activity has been termed a ‘magic bullet’ as a result of its ability to positively impact so many risk factors for chronic disease (Booth *et al.*, 2000). The positive effect gained from exercise is a consequence of the many adaptations to regular exercise, which can reduce the risk of virtually all chronic diseases simultaneously (Booth *et al.*, 2000). However, the responses to an acute bout of exercise are quite different to the responses to regular exercise (Figure 1.1).

Acute exercise is accompanied by biochemical changes including oxidative stress (Section 1.2 and 1.4). This condition is associated with the pathology of many chronic diseases and oxidative stress is generally considered to be a harmful and unwanted event. This has led to acute physical exercise being employed as a model for studying

the responses of physiological regulatory mechanisms (Sen, 2001b). Ostensibly, a transient increase in oxidative stress might initially appear to contradict the well-documented protective effect of regular exercise. However, this points towards the classical physiological concept of *hormesis*, which was fittingly described by Finkel and Holbrook (2000) in a recent review for *Nature* as ‘what won’t kill you will make you strong’. These authors proposed that the anti-ageing benefits of regular exercise are mediated by the adaptive response to regular episodes of exercise-induced oxidative stress.

The adaptations to regular exercise are associated with improved health and include changes to the musculoskeletal, cardiovascular, respiratory, immune and endocrine systems (Fentem, 1992). The magnitude of these changes will vary depending on the duration, intensity and mode of exercise, which is why it is important to be able to quantify these subcomponents of physical activity. Long term exercise training is associated with improved antioxidant defence systems in tissue such as skeletal muscle and erythrocytes (Powers *et al.*, 1999; Miyazaki *et al.*, 2001) and this has been reported to lower resting markers of oxidative stress (Niess *et al.*, 1999b; Vincent *et al.*, 1999; Miyazaki *et al.*, 2001).

Although the adoption of an active lifestyle leads to a reduction in the risk of chronic diseases it is possible that under certain conditions exhaustive exercise may be detrimental to health. In even the most well trained and therefore well adapted individuals an acute bout of demanding exercise leads to damage to lipids, proteins and DNA (Mastaloudis *et al.*, 2001; Tsai *et al.*, 2001; Fehrenbach *et al.*, 2003b; Nieman *et al.*, 2003). This is associated with an acute inflammatory response and protracted immuno-suppression (Nieman and Pedersen, 1999; Pedersen and Hoffman-Goetz, 2000). Because of these changes it has been proposed that very active individuals should consider prophylactic intervention to avoid these potentially unwanted side effects of exercise. For example, it may be appropriate to consume antioxidant supplements to enhance the antioxidant defence system, since the changes derived from regular exercise training may not be enough to suppress exercise-induced oxidative stress (Sen, 1995; Ashton *et al.*, 1998). Furthermore, this may also be very important for people who exercise infrequently as they are unlikely to be adapted to the demands of the activity and may be at even greater risk of exercise-induced oxidative stress (Davison *et al.*, 2002; Vincent *et al.*, 2005). In these situations, the use of nutritional therapies designed to reduce oxidative stress may be of direct benefit. In addition, it is noteworthy that it is only through intervention with specific nutrients that it will be

possible to determine whether a particular nutrient has a particular role and whether intervention is justified for the long-term maintenance of health.

This thesis will explore these possibilities in humans. The initial investigations that are reported examine the responses to exercise-induced oxidative stress in mononuclear cells following prolonged running. Subsequent work characterises the responses to an exogenous oxidant challenge; prior to using these two approaches in combination to determine whether an acute bout of exercise modifies the response to an exogenous oxidative challenge. These studies were designed to develop an appropriate model for the final experiment - where nutritional modulation of the responses to oxidative stress was investigated using a potentially important carotenoid (lycopene).

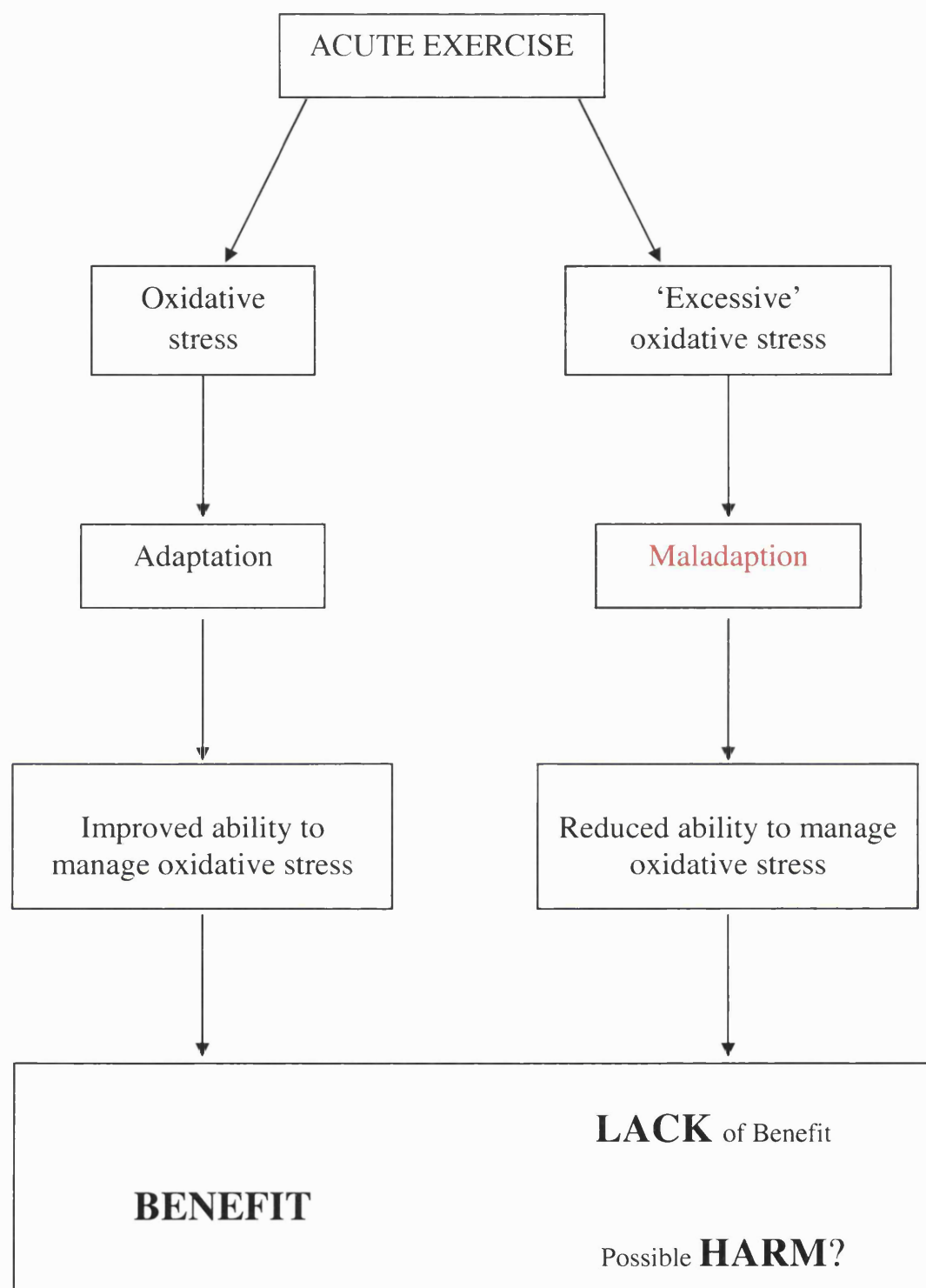


Figure 1.1 Responses to acute exercise and oxidative stress. The pathway on the left represents the response to a moderate bout of exercise and is beneficial. In contrast, a prolonged and intense period of exercise can cause excessive free radical production and may have negative consequences for tissues.

1.2 Oxidative stress

Oxygen is essential for the survival of aerobic organisms, although too much oxygen results in toxicity (Halliwell and Gutteridge, 1999). Aerobic organisms produce reactive oxygen species (ROS) during normal respiration and inflammation and are important regulators of cell function. Reactive oxygen species is the collective term for oxygen derived and non-oxygen derived free radicals (Section 1.2.1). The body is equipped with an elaborate defence system providing protection against free radical production since an increase in radical formation can cause an imbalance of the redox state of the cell, causing a situation known as 'oxidative stress.' Sies (1991) defined this as 'a disturbance of the pro-oxidant–antioxidant balance in favour of the former leading to potential damage.' In principal, molecules such as lipids, protein and DNA are susceptible to this 'potential damage.'

1.2.1 Free radical production

A free radical is an atom, group of atoms or molecule in a particular state, with one unpaired electron occupying the outer orbital (Del Maestro, 1980). This feature contributes to the very unusual chemical reactivity and physical characteristics of free radicals, allowing them to participate in chain reactions (Jenkins, 1988), initiating damage to DNA, lipids and proteins. Molecular oxygen is an example of a biradical, a species containing two unpaired electrons in its outer orbital. The reduction of O_2 to water requires four electrons which can occur by the tetravalent reduction of O_2 to water with cytochrome oxidase as the final catalyst, or by the univalent pathway. The main difference in these two processes is the highly reactive intermediate oxygen species which are produced by the four stage univalent reduction to water (Figure 1.2).

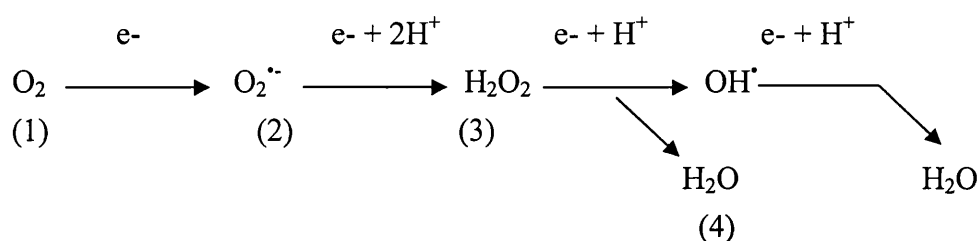
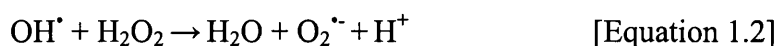


Figure 1.2 The univalent reduction of oxygen to water. (Source: Del Maestro, 1980).

From Figure 1.2, the univalent reduction of molecular oxygen produces superoxide ($O_2^{\cdot-}$) (1), hydrogen peroxide (H_2O_2) (2), and the hydroxyl radical (OH^{\cdot}) (3), before producing water (H_2O) (4). Superoxide and H_2O_2 are relatively non-toxic and can be regulated by enzymatic mechanisms present in the cellular environment, although they can also react with one another to form highly reactive OH^{\cdot} [Equations 1.1]. In addition, OH^{\cdot} and $O_2^{\cdot-}$ can also be generated through interaction with transition metals in the Haber-Weiss reactions [Equations 1.2-1.4].



Hydrogen peroxide is a non-oxygen derived radical and is a weak oxidising agent, however, it crosses membranes readily and can extend the cellular damage through its involvement in the formation of OH^{\cdot} which oxidises lipids, proteins and nucleic acids. Other non-oxygen derived radicals include hypochlorous acid ($HOCl$), singlet oxygen (1O_2) and ozone (O_3). The superoxide radical, hydroxyl radical, peroxy radicals (ROO^{\cdot}) and alkoxy radicals (RO^{\cdot}) form the oxygen-centred radicals. Free radicals are generated as a consequence of normal metabolism and are essential for the maintenance of cellular function. Superoxide is the precursor of most ROS and therefore the potential sites at which $O_2^{\cdot-}$ is produced will be reviewed.

1.2.2 Mitochondrial electron transport

The main function of mitochondria is energy production (Leeuwenburgh and Heinecke, 2001). Under normal physiological conditions, approximately 95% of oxygen consumption is reduced to water, with 2-5% forming $O_2^{\cdot-}$ (Ames *et al.*, 1995). This production of $O_2^{\cdot-}$ is described as a 'leak' with at least two sites in the electron transport chain identified; NADH-ubiquinone reductase (complex 1) and ubiquinone-cytochrome *c* reductase (complex 3) (Chance *et al.*, 1979). Fortunately, the mitochondrial membranes possess a well developed scavenger system to protect against free radical reactions (Section 1.7).

At rest (State 4 respiration), low rates of O_2 are consumed as the metabolic demands of the body are low. During this state, approximately 1-5% of the O_2 consumed produces reactive oxygen intermediates (Boveris and Chance, 1973). Resting O_2 consumption utilises approximately $3.5 \text{ ml.kg}^{-1}.\text{min}^{-1}$ and, assuming only 1% of this O_2 is converted to $O_2^{\cdot-}$ in the mitochondria, it is estimated that approximately 0.147 mole $O_2^{\cdot-}$ and 0.074 mole H_2O_2 are produced per day in state 4 respiration (Leeuwenburgh and Heinecke, 2001). In comparison, an increase in O_2 consumption causes the rate of oxidative phosphorylation to be high (State 3-ADP stimulated). The rate of leakage from lung mitochondria was found to increase proportionately to the increase in oxygen tension associated with hyperoxia, translating into an increase in $O_2^{\cdot-}$ formation (Halliwell and Gutteridge, 1999). It is generally assumed that an increase in oxygen consumption results in an increase in the production of ROS, and this is the suggested mechanism for the production of free radicals during exercise (Ji, 1999).

Oxygen consumption during exercise can increase 15 to 20-fold above resting levels (Ji, 1999) when the increase at the muscle level will be in the region of 100 to 200-fold (Sen, 1995; Halliwell and Gutteridge, 1999). Therefore, following the assumption that the percentage of oxygen produced as $O_2^{\cdot-}$ remains the same, this would result in a proportionate increase in free radical production. However, this hypothesis was challenged by Chance and colleagues (1979), who demonstrated ROS production rate in isolated mitochondria in state 3 respiration (for example, exercise) is actually lower than state 4 (rest). Heavy exercise is not equivalent to state 3 respiration and is associated with increased free radical production and oxidative stress. Therefore, the mitochondrial electron chain is not the only mechanism for increased ROS production during exercise. For example, ROS production can occur as a consequence of inner membrane damage (Davies *et al.*, 1982). Furthermore, during very intense exercise, the xanthine oxidase system can be employed increasing ROS production. However, the mitochondrial electron transport chain is the main producer of $O_2^{\cdot-}$ *in vivo* (Halliwell and Gutteridge, 1999), and this is indirectly supported by the training adaptations to mitochondrial antioxidant enzymes (Powers *et al.*, 1999) (Section 1.7).

1.2.3 Xanthine Oxidase

In situations where the rate of adenosine triphosphate (ATP) synthesis does not match the rate of ATP hydrolysis, the consequence is an insufficient availability of mitochondrial-derived ATP. This environment favours the activation of the xanthine oxidase pathway (Hellsten, 2000), which is particularly important in ROS generation in ischaemia and reperfused (I-R) cardiac muscle. When a tissue has a high energy

demand, for example during demanding exercise, ATP utilisation exceeds the rate of ATP regeneration. An accumulation of adenosine diphosphate (ADP) is prevented through the adenylate kinase reaction leading to the formation of ATP and adenosine monophosphate (AMP) from 2ADP. An increase in AMP leads to the activation of AMP deaminase forming inosine monophosphate (IMP) which is catabolised to hypoxanthine (HX), the substrate for xanthine dehydrogenase (XDH). This enzyme is found in the capillary endothelium of most tissues and exists in two forms, the XDH and the oxidase form (XO). In certain conditions, XDH is converted to XO by intracellular proteases activated by calcium ions in an irreversible reaction. Both enzymes participate in the purine degradation pathway oxidizing two reactions, HX to xanthine and xanthine to urate (UA), however, they utilise different electron acceptors. As XDH requires NAD^+ and XO requires molecular oxygen, $\text{O}_2^{\bullet-}$ formation only occurs in the XO catalysed reaction. In the XDH conversion no reactive intermediates are generated. Several inflammatory conditions activate xanthine oxidase, including atherosclerosis, rheumatic diseases and influenza (Hellsten, 2000).

A positive relationship between the peak HX concentration and change in urate of plasma following high-intensity exhaustive cycling has been demonstrated with the muscle the main source of plasma HX (Hellsten-Westling *et al.*, 1994). Similarly the xanthine oxidase pathway is activated in maximal intensity intermittent treadmill sprinting (Balsom *et al.*, 1992). In comparison, moderate intensity exercise leads to a low release of HX from muscle and therefore it is less likely that HX will accumulate in the plasma for conversion to urate (Hellsten, 1994).

1.2.4 Phagocytosis

Phagocytosis is a response to acute inflammation initiated by the migration of a variety of leukocytes to the site of injury (Conner and Grisham, 1996). Chemotactic factors released by the damaged cells attract neutrophils where they function to destroy invading pathogens. Neutrophils contain NADPH oxidase and myeloperoxidase which generate $\text{O}_2^{\bullet-}$, H_2O_2 and HOCl . Resting neutrophils use little oxygen as they have rich stores of glycogen and can produce ATP via glycolysis. However, when neutrophils phagocytose they require a marked increase in oxygen uptake. The increased oxygen uptake can be 10-20 times the 'resting' oxygen consumption and this is known as the 'respiratory burst' (Babior, 1997). The increased oxygen uptake activates an enzyme complex on the plasma membrane which in turn activates the NADPH oxidase complex. This complex contains NADPH oxidase, FAD and a *b*-type cytochrome located in the plasma inner membrane. Activation of the complex operates an electron transport chain

oxidizing NADPH in the cytosol to NADP^+ releasing two electrons that consequently reduce molecular oxygen to form $\text{O}_2^{\bullet-}$ (Cannon and Blumberg, 2000) [Equation 1.5] (Figure 1.3).

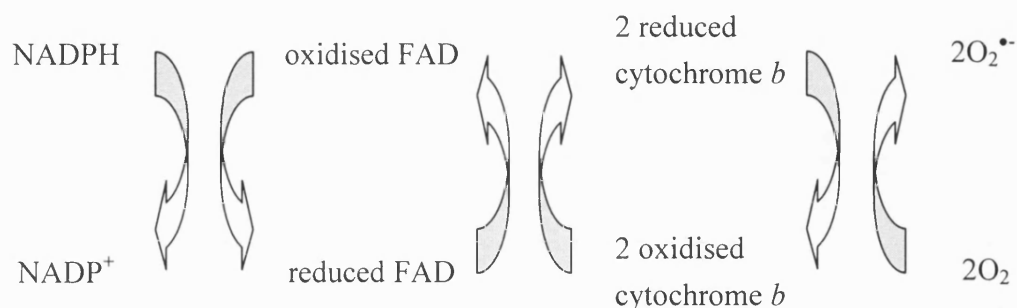


Figure 1.3 The electron transport chain within the NADPH oxidase complex. (Source: Halliwell and Gutteridge, 1999).

Dismutation of $\text{O}_2^{\bullet-}$ forms H_2O_2 , which is capable of crossing membranes. Hydrogen peroxide is toxic to bacteria, therefore causing damage directly or it can react with free iron to form OH^{\bullet} . Furthermore, HOCl is generated from $\text{O}_2^{\bullet-}$ in the presence of the enzyme myeloperoxidase (Weiss *et al.*, 1985) and can also be used to form OH^{\bullet} [Equation 1.6].



The function of phagocytes is critical in the removal of damaged tissue and prevention of bacterial and viral infection. However the cocktail of reactive species generated can also damage healthy tissue, oxidizing lipids, proteins, and DNA (Halliwell and Gutteridge, 1999). In addition, phagocyte-derived ROS may amplify inflammation via the up-regulation of genes involved in the inflammatory response, initiating the release of pro-inflammatory cytokines (Conner and Grisham, 1996) further augmenting free radical production and hence the resulting oxidative damage.

The inducible form of nitric oxide synthases (iNOS) is present in neutrophils, monocytes and T-lymphocytes (Lincoln *et al.*, 1997). Two other forms of NOS exist; neuronal NOS (nNOS) and endothelial NOS (eNOS). Certain cytokines can induce iNOS converting the amino acid L-arginine into nitric oxide (NO^{\bullet}). Superoxide reacts rapidly with NO^{\bullet} forming peroxynitrite (ONOO^-), a potent oxidising and nitrating

intermediate (Equation 1.7). Peroxynitrite can be decomposed forming peroxynitrous acid (ONOOH) and other end products such as nitrogen dioxide radicals (NO_2^\bullet) and OH^\bullet which damage tissue (Equation 1.8).



Nitric oxide has been implicated in multiple physiological processes including the regulation of blood pressure, inhibition of platelet aggregation, neurotransmission and lung vasodilation. However, excess production of NO^\bullet may cause cellular injury often resulting from the action of iNOS and its conversion into other reactive nitrogen species such as ONOO^- . The tissue injury resulting from NO^\bullet has been demonstrated in the pathology of diseases including epilepsy, stroke, rheumatoid arthritis, transplant rejection and asthma (Halliwell and Gutteridge, 1999).

1.3 Consequences of oxidative stress

Free radical production is part of normal metabolism and is generally balanced by antioxidant defence systems. When the balance is disturbed oxidative stress develops, which may cause cellular adaptation, injury or death. Non-lethal exposure to free radicals is known to induce specific stress proteins which function by protecting the cell from further oxidative challenges (Jackson *et al.*, 1998). The alternative response is cellular injury, resulting from free radical reactions with lipids, proteins and nucleic acids. The primary cellular target of oxidative stress varies depending on the cell, the type of stress imposed and the severity of the stress. Ultimately, when the cell cannot cope with the demands placed upon it, cell death ensues either as apoptosis or necrosis (Section 1.3.2). The damage caused by oxidative stress can be measured by quantifying the degree of oxidative stress encountered by a specific tissue, although there are many different techniques employed and no gold standard marker of oxidative stress has yet been identified (Griffiths *et al.*, 2002).

1.3.1 Markers of oxidative stress

A marker of oxidative stress should be a major product of oxidative modification that is implicated in the development of disease and is unaffected by normal cell processes (Griffiths *et al.*, 2002). In addition, a valid marker should be a stable product that can be

determined by an assay that is specific, sensitive, reproducible and robust (Griffiths *et al.*, 2002). As oxidants have the potential to damage membranes, proteins and DNA, suitable markers are often the biomolecules formed as a consequence of this damage (hence the term 'biomarker'). Despite this, many of the available detection methods and end products are not sensitive enough or specific, giving rise to artifactual oxidation products making them unsuitable for *in vivo* application. The conflicting research available in the literature that has investigated oxidative stress *in vivo* demonstrates the difficulty encountered in quantifying oxidative stress (Han *et al.*, 2000). For example, total lipid peroxidation is difficult to definitively measure as a result of the wide range of products generated, with lipid hydroperoxides, aldehydes, conjugated dienes, volatile hydrocarbons, and F2-isoprostanes all being potential options. In addition, using one biomarker to evaluate lipid peroxidation can be misleading because the biomarkers are the products of different stages of lipid peroxidation and this could explain why some laboratories have reported oxidative stress induced by a particular stressor and others have not.

Lipid peroxidation products tend to be very unstable and extra caution needs to be taken during processing and extraction. A discussion on this topic is beyond the scope of this literature review and therefore the reader is referred to Gutteridge (1995). Several investigations have demonstrated an increase in lipid peroxidation following damaging exercise (Maughan *et al.*, 1989) and prolonged exercise (Mastaloudis *et al.*, 2004a) with antioxidant supplementation capable of attenuating the increase in lipid peroxidation (Meydani *et al.*, 1993) and inflammation associated with exercise (Cannon *et al.*, 1991).

The only method that can directly detect free radical species is electron spin resonance (ESR) using spectroscopy to detect unpaired electrons. Free radicals have a very short life time and in order to detect free radicals in biological systems, spin traps must be used to ensure specific and stable products. Spin traps which are commonly used are nitrones, such as phenyl-tert-butyl nitron (PTBN) and 5, 5-dimethyl-1-pyrroline N-oxide (DMPO). This method has been used to demonstrate exercise-induced oxidative stress in skeletal muscle (Davies *et al.*, 1982; Jackson *et al.*, 1985) and human plasma (Ashton *et al.*, 1998). Although this technique directly detects free radicals, only a few radical species have been identified. Furthermore, an increase in a single free radical species does not provide information relating to oxidative stress. An increase in the detection of one free radical species may be accompanied by decreases in other free radical species and, therefore, this does not provide information relating to the overall concentration of free radicals or whether this has been accompanied by a compensatory increase in

antioxidant molecules. Consequently, this method does not measure redox status or quantify oxidative stress.

There are many techniques available to detect oxidatively modified products of DNA and proteins. The damage caused by ROS can lead to DNA strand breaks, DNA crosslinks and base pair oxidation. In exercise studies the measurements of oxidised base pairs 8-oxoguananine (8-oxoG) or 8-deoxyguanosine (8-oxodG) are widely used and can be detected using high performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC-MS), and enzymatic assays. Variation in values among these different techniques is common and is probably the consequence of artifacts generated during extraction and analysis. Several investigations have reported lymphocyte DNA damage as a result of exercise, hypoxia and H₂O₂ treatment (Hartman, 2000; Moller *et al.*, 2001; Rothfuss *et al.*, 2001; Tsai *et al.*, 2001).

The oxidative modification of proteins can be assessed by the measurement of thiol/disulphide redox status, protein carbonyls and oxidized amino acids. A wide variety of methods exist for the measurement of reduced glutathione (GSH) in biological systems, although the measurement of the GSH/GSSG (glutathione disulphide) redox status is a more specific marker of oxidative stress than GSH alone. When there is excessive production of ROS that exceeds the recycling capacity of glutathione reductase, GSSG levels will rise resulting in a decrease in the GSH/GSSG ratio. The major limitation of this marker is the auto-oxidation of GSH to GSSG during sample processing providing discrepancies in values. Exercise has been shown to increase levels of GSH and GSSG without changing the GSH/GSSG ratio in rat skeletal muscle (Ji *et al.*, 1992) as well as reduce whole blood GSH concentration and increase GSSG following eccentric exercise (Goldfarb *et al.*, 2005). Additionally, eccentric exercise has been shown to induce oxidative stress, assessed by increased plasma protein carbonyl concentration, without alterations in the blood glutathione status (Lee *et al.*, 2002). However, it should be pointed out that the use of protein carbonyls as a biomarker of oxidative stress is controversial because protein carbonyls can be formed by reactions that are not specific to oxidative stress (Han *et al.*, 2000).

It is clear that the measurement of oxidative stress and damage in biological systems is a difficult task. Rather than attempting to determine a specific product of oxidative modification, it may be possible to use the altered expression of oxidant-sensitive genes as tools to determine whether there has been a disturbance in redox status (Offord *et al.*, 2000). The most appropriate candidate at the present time appears to be Haem Oxygenase-1 (HO-1) (Offord *et al.*, 2000; Griffiths *et al.*, 2002). Haem oxygenase-1 is

often described as an oxidant stress protein (Applegate *et al.*, 1991; Abraham *et al.*, 1996). The current criteria used to determine whether a biomarker is valid include (i) specificity (both biological in source, and chemical in terms of analysis) (ii) knowledge of its functional significance (iii) stability (iv) an assay which is specific, sensitive and reproducible (v) accessibility in a target or valid surrogate tissue (Griffiths *et al.*, 2002). Haem oxygenase-1 potentially satisfies all these criteria and has been used as a biological marker of oxidative stress *in vitro* (Tyrrell and Basu-Modak, 1994). The role, function and relevance of HO-1 is discussed later in this review (Section 1.5).

1.3.2 Cell death

Exposure of cells to severe oxidative stress leads to cell death. This can occur by two mechanisms; apoptosis (programmed cell death) and necrosis. Treatment with high concentrations (mM) of H₂O₂ induce cell death by necrosis while lower concentrations induce apoptosis (Hampton and Orrenius, 1997; Antunes and Cadenas, 2001). This suggests that apoptosis is a mechanism that has developed to sacrifice individually damaged cells for the sake of the organism. Necrosis occurs as a result of trauma or injury and has specific and different morphological and biochemical features to those observed in apoptosis (Table 1.2). Although apoptosis is a mode of cell death, it is generally accepted that this strategy maintains the dynamic balance and equilibrium of living systems, occurring as a normal mechanism in development and homeostasis (Zhivotovsky *et al.*, 1999).

Table 1.2 Distinguishing features of apoptosis and necrosis

Apoptosis	Necrosis
Physiological features	
Physiological/Pathological	Pathological
Single cells	Groups of cells
Phagocytosis by other cells	Phagocytosis by macrophages
No inflammatory response	Inflammatory response
Morphological features	
Cell shrinkage	Swelling of the cell and lysis
Membrane blebbing	Loss of membrane integrity
Formation of apoptotic bodies	No vesicle formation, complete lysis
Intact organelles	Swelling of organelles
Biochemical features	
Gene activation and protein synthesis	Loss of regulation of ion homeostasis
ATP dependent (active)	No energy requirement (passive)
Non-random DNA fragmentation	Randomised digestion of DNA
Pre-lytic DNA fragmentation	Post-lytic DNA fragmentation
Phosphatidylserine (PS) exposure	Absent
Activation of caspases	Absent

(Adapted from Zhivotovsky *et al.*, 1999).

Apoptotic cells shrink and, if relevant, they will retract from their neighbouring cells. The process is followed by condensation of the chromatin at the nuclear membrane. The membrane forms blebs and vesicles and finally there is nuclear fragmentation and the formation of apoptotic bodies (Wyllie, 1980). These 'bodies' contain fragments of nuclear chromatin and can be phagocytosed, preventing the spillage of cellular contents. These characteristics of apoptosis reflect the activation of a cascade of cysteine proteases known as caspases. Caspases are cysteine-containing, aspartic acid-specific proteases which exist as zymogens in the cytoplasm, intermembrane space of

mitochondria and the cell nucleus (Nicholson and Thornberry, 1997). Apoptosis is regulated by an extrinsic and an intrinsic pathway. In the extrinsic pathway, cell surface receptors are activated which recruit caspases; whereas the intrinsic pathway depends on the release of cytochrome *c* from mitochondria into the cytoplasm as a result of changes in the membrane permeability (Green and Reed, 1998). The latter pathway is suggested to occur in H₂O₂-induced apoptosis with cytochrome *c* binding to apoptotic protease activating factor (Apaf-1), forming an apoptosome (Chandra *et al.*, 2000). The apoptosome forms a complex with procaspase-9, converting it to its active form, initiating a caspase cascade. The formation of the protein complex between Apaf-1, cytochrome *c* and procaspases requires energy, hence, apoptosis is seen as an active form of cell death (Stoneman and Bennett, 2004).

Apoptosis can also be initiated by changes in the dysregulation of Ca²⁺ metabolism. Increases in Ca²⁺ can activate Ca²⁺-dependent endonucleases in the cell nucleus, causing DNA fragmentation (Wyllie, 1980). Certain oxidants open a permeability transition pore (PTP) between the inner and outer mitochondrial membranes, which becomes activated by a high intracellular Ca²⁺ concentration in the mitochondrial matrix and oxidative stress, resulting in the release of cytochrome *c* (Orrenius *et al.*, 2003). In healthy cells, phospholipids are distributed asymmetrically with phosphatidylserine (PS) located in the inner plasma membrane, however, in apoptotic cells PS translocates to the cell surface, providing a signal for phagocytosis. Currently, it is recognised that PS exposure in apoptosis is Ca²⁺ dependent, demonstrating the role of Ca²⁺ in apoptotic cell death (Orrenius *et al.*, 2003).

Cell death is regulated by the Bcl-2 family of proteins, consisting of pro- and anti-apoptotic proteins. The Bcl-2 protein is localised to the mitochondria, endoplasmic reticulum and nuclear membrane. Bcl-2 family proteins are known to play a pivotal role in the induction of caspase activation and in the regulation of apoptosis. Bcl-2 has been shown to inhibit oxidative stress-induced apoptosis supporting the notion that this protein possesses antioxidant properties (Hockenbery *et al.*, 1993). Furthermore, over-expression of Bcl-2 was found to reduce UVA-induced apoptosis which was associated with a reduced expression of the oxidant-responsive gene HO-1 (Pourzand *et al.*, 1997). The induction of HO-1 forms a major part of this thesis and will be discussed in section 1.5. The role of Bcl-2 proteins in apoptosis is beyond the scope of this literature review and the reader is referred to Burlacu (2003).

The different characteristics of apoptosis and necrosis have resulted in the development of a wide variety of techniques to assess cytotoxicity. Evidence of apoptosis and

necrosis is discussed in later sections of this review in relation to exercise, H₂O₂ treatment, HO-1 expression and antioxidant supplementation (Sections 1.4.2, 1.6.2, and 1.6.3).

1.4 Exercise-induced oxidative stress

Acute exercise places the body under a range of different stressors, with several different exercise protocols demonstrating an induction of oxidative stress in both skeletal muscle (Jackson, 2000), blood leukocytes (Niess *et al.*, 1998; Suzuki *et al.*, 1999), and plasma (Lee *et al.*, 2002; Sacheck *et al.*, 2003; Vincent *et al.*, 2004). The magnitude of oxidative stress induced by exercise is related to the mode of exercise, intensity and duration as these factors contribute to the different mechanisms capable of producing free radicals (Jackson, 2000; Konig *et al.*, 2001).

During and following a bout of exercise many biochemical reactions take place which can lead to the formation of free radicals (Figure 1.4). As mentioned previously, exercise-induced increases in oxygen consumption amplify free radical production via the electron transport chain. Furthermore, splanchnic blood flow is reduced during demanding exercise (Clausen, 1977), and this relative ischaemia may lead to increased superoxide production upon reperfusion. The reduced blood flow may also cause alterations in gastro-intestinal function, increasing the permeability of the gut wall allowing bacterial lipopolysaccharide (LPS) to translocate from the gut into the plasma, causing endotoxaemia (Bosenberg *et al.*, 1988). Plasma LPS has been reported to increase significantly in individuals immediately following a long distance triathlon (Bosenberg *et al.*, 1988; Jeukendrup *et al.*, 2000) and short-term maximal aerobic exercise (Ashton *et al.*, 2003). Additionally, the xanthine oxidase pathway is activated in intense exercise, leading to increases in uric acid production following exercise (Balsom *et al.*, 1992). Exercise increases catecholamine levels (Muir *et al.*, 1984), which can auto-oxidise generating free radicals. Changes in blood leukocyte levels occur during and in the hours following exercise and this inflammatory response can increase free radical production and augment oxidative stress (Suzuki *et al.*, 1996; Morozov *et al.*, 2003). Therefore, exercise provokes a number of pathways that have the potential to overwhelm antioxidant defences and induce oxidative stress.

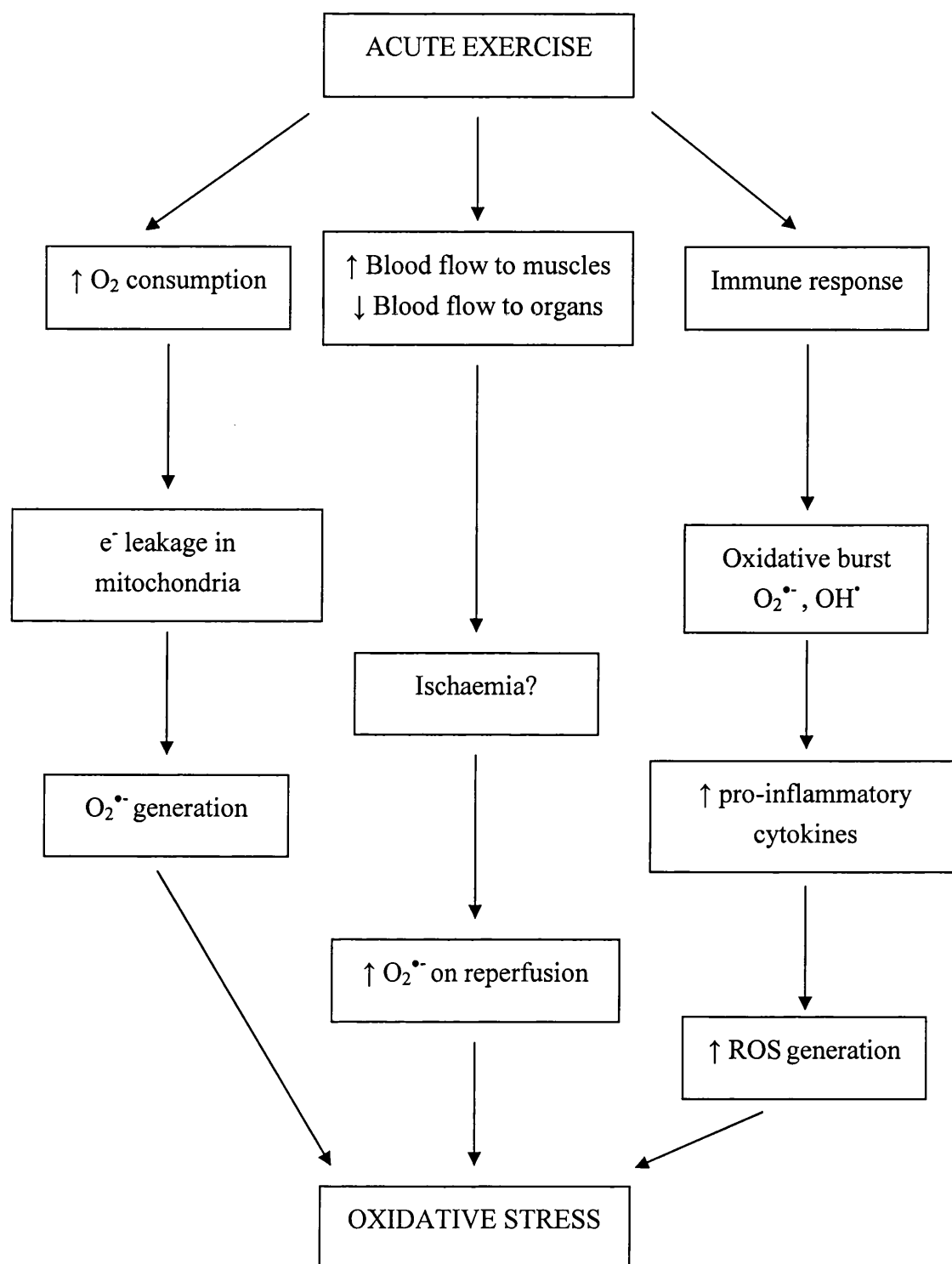


Figure 1.4 Proposed mechanisms for the induction of oxidative stress in non-damaging acute exercise.

Exercise-induced oxidative stress has been assessed using indirect markers of free radical production, such as lipid peroxidation (Maughan *et al.*, 1989), protein oxidation (Radak *et al.*, 1997), thiol status (Radak *et al.*, 2004) and DNA damage (Hartman, 2000). The use of ESR provides a direct measurement of free radical concentration in biological tissue, although some investigations have not been able to definitively identify the free radical species detected (Ashton *et al.*, 1998). The first investigation using ESR to detect oxidative stress was conducted in muscle homogenate of rat after an acute bout of treadmill running to exhaustion (Davies *et al.*, 1982). The free radical species reported to increase was proposed to be a semi-quinone, providing evidence that ROS were generated as a result of exercise. In addition, damaging exercise consisting of repeated muscular contractions was reported to increase the intensity of the ESR signal by 70%, leading the authors to conclude free radical production may play a role in muscle damage associated with intense contractile activity (Jackson *et al.*, 1985). The free radical species that was detected was believed to be a semi-quinone type radical.

Exhaustive aerobic exercise was demonstrated to increase free radical production in human plasma, detected using ESR, and this was temporally associated with increased lipid peroxidation post-exercise (Ashton *et al.*, 1998). Recently, the first evidence for oxygen- (alkoxyl/peroxyl radical) or carbon- centred (alkyl radical) free radical outflow from an active muscle bed in humans was reported (Bailey *et al.*, 2003). In this investigation, individuals completed incremental exercise encompassing 25 and 70% of their previously established maximal work rate using a single-leg knee extensor model, establishing support for the production of free radicals in humans participating in exercise.

As mentioned previously, the magnitude of oxidative stress induced by exercise is dependent on several factors, although few investigations have compared different exercise protocols to determine which protocols generate a greater level of oxidative stress. Intermittent exercise performed at the same average work rate as continuous exercise has been associated with increased physiological strain (Edwards *et al.*, 1973). It is known that oxygen consumption of continuous and intermittent aerobic exercise and contractile activities differs, therefore the production of free radicals in these different exercise protocols should differ. However, as illustrated in Figure 1.4, several biochemical mechanisms produce free radicals in exercise and different protocols will activate any number of sources.

In a study examining oxidative stress induced by aerobic or isometric exercise both protocols demonstrated increases in plasma lipid hydroperoxides, protein carbonyls and

antioxidant capacity, although the degree of change in each measure differed between protocols (Alessio *et al.*, 2000). Aerobic exercise resulted in a 14-fold change in oxygen consumption compared to only two-fold with isometric exercise and had a greater increase in protein carbonyl concentration immediately post-exercise (67% vs. 12%, respectively). However, lipid hydroperoxide concentration following the isometric exercise increased 36% from pre-exercise values compared to 24% in the aerobic exercise protocol. This investigation suggests that although both protocols are capable of inducing oxidative stress, the nature of the oxidative stress may differ due to the very different metabolic demands of the two exercises. Similarly, 30 min downhill running was associated with elevated plasma MDA concentrations whereas running on a flat for the same period of time did not alter plasma MDA concentration and did not induce oxidative stress (Close *et al.*, 2004). The responses to exercise are made even more difficult to predict since the inflammatory response to exercise changes with different exercise protocols (Natale *et al.*, 2003) and this may be a possible mechanism for differences in the degree of oxidative stress.

1.4.1 Inflammation

The inflammatory response associated with exercise represents a mechanism for the production of free radicals. Intense exercise causes an increase in total blood leukocyte counts during and following exercise, with changes to leukocyte subpopulations (Hoffman-Goetz and Pedersen, 1994). The change in leukocyte number is largely the result of the increase to the neutrophil population with a smaller increase in monocytes. During exercise, lymphocyte count increases (Field *et al.*, 1991; Natale *et al.*, 2003) with increased recruitment of natural killer (NK) cells, B cells, and T cells to the blood (Nieman *et al.*, 1991). The increase in leukocyte number has been reported to be related to exercise intensity, increasing in an intensity-dependent manner (Miles *et al.*, 2003; Quindry *et al.*, 2003). The neutrophil count remains elevated several hours following exercise, whereas the decline in monocyte and lymphocyte counts occurs earlier, at one or two hours post-exercise.

Activated neutrophils are capable of producing $O_2^{\cdot-}$ (Section 1.2.4), and can generate HOCl in the presence of myeloperoxidase, destroying invading bacteria and removing damaged tissue. Several groups have reported an increase in neutrophil activation following exercise (for review see Woods *et al.*, (1999)) demonstrated by the presence of myeloperoxidase in the plasma (Morozov *et al.*, 2003; Suzuki *et al.*, 2003). Although the removal of damaged tissue is a desirable function, neutrophils also secrete pro-inflammatory cytokines and other destructive agents which can lead to further

augmentation of ROS production and inflammation (Cannon *et al.*, 1991). Cytokines are secreted by and influence the action of most cells in the body. The inflammatory response is regulated by pro-inflammatory and anti-inflammatory cytokines. Increases in the pro-inflammatory cytokines interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor- α (TNF- α) have been reported following exercise (Cannon *et al.*, 1991; Niess *et al.*, 1999b; Moldoveanu *et al.*, 2000). Infusion of IL-6 at levels equivalent to those observed in strenuous exercise resulted in increased plasma concentrations of anti-inflammatory cytokines [interleukin-1 receptor antagonist (IL-1ra) and interleukin-10 (IL-10)] and increased cortisol concentration altering neutrophil and lymphocyte counts (Steensberg *et al.*, 2003).

A recent review examined the effects of glucocorticoid hormones and catecholamines in their ability to alter the function of leukocytes and subsequent release of cytokines (Padgett and Glaser, 2003). Evidence of this role was reported as early as 1984 with the infusion of intravenous adrenaline increasing circulating leukocyte number in the lung (Muir *et al.*, 1984), although much smaller increases than those observed following exercise were documented. Similarly, the redistribution of lymphocyte subsets caused by exercise has been shown to be the result of adrenaline-induced changes in beta-adrenergic receptors (Frey *et al.*, 1989). The role of adrenalin was supported by Field and colleagues (1991) observing a positive correlation between total leukocyte counts following a short exhaustive aerobic exercise protocol and plasma adrenalin concentration. This relationship suggested a causal effect was possible. However, cortisol levels change during exercise and an increase in this hormone may explain the decline in lymphocyte number in the hours following exercise (Nieman *et al.*, 1993), as cortisol causes NK cells to exit the circulation in large numbers. The alternative proposed mechanism for the decline in lymphocyte cell number following exercise is lymphocyte apoptosis.

1.4.2 Exercise-induced apoptosis

Strenuous exercise results in increased glucocorticoid secretion and ROS production, and has been shown to increase the intracellular calcium concentration in lymphocytes (Mooren *et al.*, 2001). These signals are known to induce apoptosis (Green and Reed, 1998). The oxidative damage caused by exercise could contribute to lymphocyte damage resulting in apoptosis (Azenabor and Hoffman-Goetz, 1999). It is speculated that exercise-induced apoptosis is a normal regulatory process that serves to remove certain damaged cells without a pronounced inflammatory response, thus ensuring optimal body function (Phaneuf and Leeuwenburgh, 2001). This has led authors to

postulate that lymphocyte apoptosis could be the reason for the decline in lymphocyte count following exercise (Mars *et al.*, 1998; Mooren *et al.*, 2002).

It is possible that changes in cortisol alone may affect lymphocyte apoptosis. Indeed, *in vitro* exposure to corticosterone at physiological concentrations ($\leq 450 \text{ ng.ml}^{-1}$) observed after a moderate exercise stress induces apoptosis in thymocytes and necrosis in both thymocytes and splenocytes (Hoffman-Goetz and Zajchowski, 1999). Interestingly, investigations examining lymphocyte apoptosis in mice have reported contradictory results with lower apoptosis reported two hours following submaximal treadmill running despite elevated cortisol levels (Hoffman-Goetz *et al.*, 1999) and higher apoptosis observed 24 h following treadmill running for the same duration (Hoffman-Goetz and Quadrilatero, 2003). The difference in these findings is likely to be the result of different time points used for analysis. The administration of antioxidants was reported to attenuate lymphocyte apoptosis following exercise in mice (Lin *et al.*, 1999; Quadrilatero and Hoffman-Goetz, 2005), suggesting that oxidative stress was the cause of exercise-induced apoptosis.

Although there is some evidence that lymphocyte apoptosis may be the mechanism for lymphocytopenia (Mars *et al.*, 1998), some research suggests that this is not the case. Steensberg and colleagues (2002) asked participants to complete a strenuous treadmill protocol running at 75% $\dot{V}O_{2\text{max}}$ for 2.5 h. Lymphocyte count increased after 30 min of running and significantly declined by 50% one hour post-exercise, remaining at this level at the 2 h post-exercise time point. Although the percentage of apoptotic cells increased by 60% at 2 h post-exercise, the total number of circulating apoptotic lymphocytes did not change in relation to exercise, indicating that the cells leaving the circulation were not apoptotic (Steensberg *et al.*, 2002).

The training status of individuals has been reported to affect the basal level of apoptosis observed in lymphocytes, with highly trained people exhibiting a higher resting level of apoptotic cells and a decline in lymphocyte apoptosis 24 h following a marathon (Mooren *et al.*, 2004). In contrast, 'badly trained' individuals had a lower basal level of apoptosis and increased apoptosis 24 h following a marathon. The effects of endurance training are known to attenuate exercise-induced oxidative stress (Miyazaki *et al.*, 2001) and, therefore, the reduced oxidative stress post-marathon could result in the attenuation of apoptosis in highly trained individuals. Furthermore, regular exercise is known to affect the expression of protective proteins in various tissues (Powers and Sen, 2000) and this may affect the basal level of apoptosis in lymphocytes.

1.4.3 Expression of protective proteins

Acute or unaccustomed exercise results in muscle damage (Pyne, 1994). However, it has been shown that training adaptations occur within muscle, leading to increased levels of various enzymes that improve protection against muscle damage and oxidative stress (Powers *et al.*, 1999). Levels of superoxide dismutase (SOD) and SOD activity have been reported to increase following acute exercise in skeletal muscle and leukocytes in both human and animal studies (Suzuki *et al.*, 2000; McArdle *et al.*, 2001; Tauler *et al.*, 2003) supporting the hypothesis that protection against oxidative stress is via increased antioxidant defences. Indeed, oxidative stress has also been related to the up-regulation of specific and protective proteins following acute exercise (Niess *et al.*, 1999b; Febbraio and Koukoulos, 2000; Fehrenbach *et al.*, 2000a; Fehrenbach *et al.*, 2000b; Khassaf *et al.*, 2001; McArdle *et al.*, 2001; Powers *et al.*, 2001; Fehrenbach *et al.*, 2003a). The up-regulation of heat shock proteins (HSPs) has been related to the attenuation of tissue damage induced by subsequent exposure to oxidative stress and other forms of stresses (McArdle *et al.*, 2004). Similarly, the up-regulation of the oxidant-responsive gene HO-1 in muscle and leukocytes has been reported following strenuous exercise and has been used as a marker of oxidative stress (Essig *et al.*, 1997; Niess *et al.*, 1999b; Pilegaard *et al.*, 2000; Thompson *et al.*, 2005). The use of this enzyme as a marker of oxidative stress is related to its functional significance with the induction of HO-1 being associated with protection against ischaemia-reperfusion injury (Zhang *et al.*, 2004), oxidative stress (Yachie *et al.*, 1999), inflammation (Nath *et al.*, 2001), transplant rejection (Soares *et al.*, 2001), and apoptosis (Choi *et al.*, 2004). The importance of HO-1 is discussed in the following section, where further evidence of the relationship between HO-1 expression and exercise will be examined (Section 1.5.3).

1.5 Haem oxygenase-1 (HO-1)

The enzyme haem oxygenase (HO) (E.C. 1.14. 99.3) was identified in 1968 and described as a 'hepatic microsomal mixed function oxygenase' (Tenhunen *et al.*, 1968;1969) confirming earlier observations by Wise and Drabkin (1964) of the *in vitro* degradation of haem to biliverdin. Despite the late discovery of this enzyme its presence can be traced back centuries, as it is a distinct visible colourimetric biological process. When a bruise appears on the skin it is a black/purple colour and, as haem catabolism occurs, the colour of the bruise changes from black (the colour of haem) to green (the colour of biliverdin) and finally to yellow (the colour of bilirubin). The different colours represent the different products of haem catabolism with HO-1 providing the rate-limiting step of this reaction.

In mammals, three isoforms of haem oxygenase have been identified; HO-1, HO-2, and HO-3 (Maines, 1997), which are the products of different genes. Their localisation and characteristics differ and this leads to variations in their inducibility and resulting function (Table 1.3). The molecular weight of these enzymes is approximately 32, 36 and 33 kDa, respectively. Haem oxygenase-1 is the most widely distributed isoform throughout the body tissues and is most concentrated in the spleen and liver where haem degradation of aged red blood cells occurs.

Table 1.3 Characteristics of the isoforms of HO

Characteristic	HO-1	HO-2	HO-3
Major function	Haem degradation	Haem degradation	Haem binding
Inducibility	Highly inducible Diverse conditions	Constitutive	Uninducible
Localisation	Ubiquitous	Mainly brain and testes	Ubiquitous

(Adapted from Elbirt and Bonkovsky, 1999).

1.5.1 Role and function

The degradation of haem to biliverdin is dependent on the enzyme activity of HO. The action of biliverdin reductase converts biliverdin to bilirubin, which has been shown to possess *in vitro* antioxidant properties (Stocker *et al.*, 1987). Equimolar amounts of iron and carbon monoxide (CO) are also released from the breakdown of haem (Figure 1.5). The complex of catalytically active iron is referred to as haem, requiring eight molecules each of glycine and succinyl CoA to synthesise one molecule of haem (Galbraith, 1999). The function of haem is essential to aerobic metabolism because active iron can donate electrons as it is in a reduced ferrous state and has a high affinity for molecular oxygen. This process allows for the transport of oxygen via haemoglobin (Hb), storage of oxygen via myoglobin (Mb), cellular respiration (cytochromes) and destruction of oxygen free radicals. Free haem is a pro-oxidant and therefore the induction of HO is essential to restore cellular oxidant to antioxidant ratio by removal of the pro-oxidant, haem, from the cell (Stocker *et al.*, 1987). Cellular damage produced by ROS is enhanced in the presence of even small amounts of haem (Fogg *et al.*, 1999) although the presence of the iron-sequestering protein ferritin improves the antioxidant status of

the cell. Ferritin is induced as a protective measure in response to free iron and therefore enhances cellular antioxidant status.

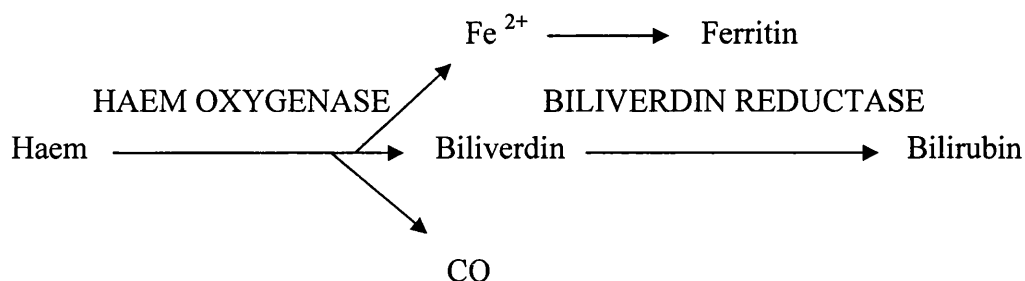


Figure 1.5 Haem degradation catalysed by HO.

The generation of bile pigments from the degradation of haem contributes to the antioxidant status of the cell, as it is known to be a very efficient scavenger of free radicals (Stocker *et al.*, 1987; Neuzil, 1993). Bilirubin is a potent antioxidant and plays a role in the cytoprotection offered by HO (Hayashi *et al.*, 1999; Novotny and Vitek, 2003). Vile and Tyrrell (1993) demonstrated a 4-fold increase in HO-1 activity was coupled to a 2-fold increase in ferritin levels following ultraviolet A irradiation. The activation of HO-1 metabolises the pro-oxidant haem molecule, and as a consequence the concentration of non-haem 'free' iron is increased (Fogg *et al.*, 1999). This causes the induction of ferritin, which reduces the oxidant state of the cells by sequestering free iron. Therefore, ferritin up-regulation decreases the availability of free iron, restricting iron-catalyzed free radical reactions during periods of subsequent oxidative stress (Vile and Tyrrell, 1993). According to Fogg and co-workers (1999) the degradation of haem by HO may be an adaptive response to oxidant stress, which is due to its dual antioxidant function by (i) preventing free haem participating in pro-oxidant reactions (Keyse and Tyrrell, 1989) and (ii) by the generation of biliverdin and bilirubin.

1.5.2 Induction of HO-1

Haem oxygenase-1 has been described as a heat shock protein (Shibahara *et al.*, 1987; Kitani *et al.*, 1993) as well as a stress protein, induced by oxidative damage (Keyse and Tyrrell, 1987;1989). Haem oxygenase-1 expression is up-regulated in the presence of oxidants and this induction appears to be a protective response. The cellular protection offered by HO-1 is evident against a wide range of oxidant stresses both *in vitro* and *in vivo* (Applegate *et al.*, 1991; Nath *et al.*, 1992; Vile *et al.*, 1994; Lee *et al.*, 1996) (Table 1.4). The induction of HO activity is affected by the cellular redox potential and

the metabolic fate of haem, and consequently this enzyme has the potential to become either a pro- or antioxidant (Ryter and Tyrrell, 2000).

Table 1.4 Inducers of HO-1 expression

Inducer	Reference
Cytokines	Cantoni <i>et al.</i> , (1991) Fernandez <i>et al.</i> , (2003)
Endotoxin	Wiesel <i>et al.</i> , (2000) Vicente <i>et al.</i> , (2003b)
Glutathione depletion	Lautier <i>et al.</i> , (1992) Ewing <i>et al.</i> , (1993)
Haem	Abraham <i>et al.</i> , (1995) Nath <i>et al.</i> , (2001)
Heavy metals	Alam <i>et al.</i> , (1994)
Hyperbaric oxygen treatment	Rothfuss <i>et al.</i> , (2001)
Hyperoxia	Lee <i>et al.</i> , (1996) Otterbein <i>et al.</i> , (1999)
Hypoxia	Panchenko <i>et al.</i> , (2000) Motterlini <i>et al.</i> , (2000)
Hydrogen peroxide	Keyse <i>et al.</i> , (1987; 1989)
Nitric oxide	Foresti <i>et al.</i> , (1997) Motterlini <i>et al.</i> , (2002)
Ultraviolet A	Keyse <i>et al.</i> , (1987; 1989)

Free radicals play a role as intracellular messengers and redox-based regulation of gene expression plays a fundamental mechanism in cell biology (Sen, 2001a). The induction of HO-1 has been shown to be regulated by redox signalling, involving the activation of mitogen-activated protein kinases (MAP kinases) (Ryter and Choi, 2002). There is evidence that the c-Jun N-terminal kinase (JNK) pathway (Morse *et al.*, 2003), p38 MAP kinase pathway and extra cellular signal-regulated kinase (ERK) pathways mediate the regulation of HO-1 (Elbirt *et al.*, 1998; Lee and Chau, 2002). In addition, DNA binding sites for oxidative stress-responsive transcription factors such as nuclear factor (NF)- κ B and activator protein-1 (AP-1) have been identified in the promoter region of HO-1 (Choi and Alam, 1996). Furthermore, the promoter region of HO-1 has been shown to be functionally active in response to various inducers such as UV irradiation, cadmium, H₂O₂ and haem (Tyrrell *et al.*, 1993). The recent discovery of the haem-binding factor Bach-1 furthered the understanding of HO-1 regulation with Bach-1 acting in a simple feedback loop directly sensing haem levels (Sun *et al.*, 2002). The expression of HO-1 and Bach-1 has been shown to be inversely related, with

inducers of Bach-1 causing repression of HO-1 (Kitamuro *et al.*, 2003). It is postulated that the regulation of HO-1 activity may be a therapeutic strategy for a number of inflammatory and disease conditions (Immenschuh and Ramadori, 2000; Alcaraz *et al.*, 2003). Clearly, it is important that the signal transduction pathways inducing HO-1 are understood.

The physiological importance of HO-1 has been highlighted in investigations examining the role of HO-1 deficiency. Studies using the targeted deletion of HO-1 in mice demonstrated an accumulation of hepatic and renal iron which was associated with oxidative damage and chronic inflammation (Poss and Tonegawa, 1997a). Furthermore, cultured embryonic fibroblasts lacking the gene were hypersensitive to cytotoxicity caused by haemin and H₂O₂ treatment (Poss and Tonegawa, 1997b). In 1999, the first case of human HO-1 deficiency was reported demonstrating a similar phenotype to knock-out mice, suffering from growth failure, anaemia, tissue iron deposition, leukocytosis, and increased sensitivity to oxidants (Yachie *et al.*, 1999). Additionally, accumulating evidence supports a role for HO-1 in preventing rejection of transplanted organs with exposure of the donor graft to CO increasing HO-1 expression in this organ as well as playing a pivotal role in suppression of the immune response to the new graft (Soares *et al.*, 2001; McDaid *et al.*, 2005). Recently, a link between IL-10 expression and HO-1 induction was demonstrated in macrophages *in vitro* in response to LPS stimulation, associated with down-regulation of pro-inflammatory cytokines (IL-1 β , TNF- α) and up-regulation of IL-10 (Otterbein *et al.*, 2000). Evidence supporting a role of HO-1 in the anti-inflammatory function of IL-10 was observed in mice with increased expression of HO-1 protein following intraperitoneal administration of IL-10 (Lee and Chau, 2002), providing an alternative mechanism for HO-1 induced protection. Studies have shown protection against atherosclerosis independently through increased HO-1 expression (Yet *et al.*, 2003) and IL-10 expression (Mallat *et al.*, 1999). Taken together, these investigations reveal a role for HO-1 induction as an adaptive mechanism protecting cells from oxidative damage during stress.

The production of free radicals and oxidative stress contributes to the pathology of many chronic diseases, with increased HO-1 expression associated with conditions such as asthma (Kitada *et al.*, 2001), atherosclerosis (Yet *et al.*, 2003), and hypertension (Wiesel *et al.*, 2001). In contrast, decreased levels of HO-1 mRNA have been found in individuals suffering from diabetes (da Silva *et al.*, 1997) and Alzheimer's disease (Ishizuka *et al.*, 2002). The ability of individuals to respond strongly in terms of up-regulating HO-1 may be an important endogenous protective factor, although there seems to be large degree of inter-individual variation. The presence of a microsatellite

polymorphism in the promoter region of the gene known as a (GT) n repeat was first reported by Yamada (2000) demonstrating that individuals with long (GT) n repeats had a higher risk of emphysema caused by cigarette smoking. Furthermore, lymphoblastoid cells from individuals with short (GT) n repeats were more resistant to oxidant-induced apoptosis than those with long (GT) n repeats (Hirai *et al.*, 2003). This provides evidence that polymorphism of the HO-1 gene is associated with the strength of anti-apoptotic effects of HO-1, resulting in an association with susceptibility to oxidative stress-mediated diseases. The effect of the HO-1 promoter polymorphism and the resulting implications to diseases has been recently comprehensively reviewed (Exner *et al.*, 2004).

1.5.3 HO-1 induction with exercise

It is accepted that acute exercise causes oxidative stress although as mentioned previously, there is no criterion standard marker for the assessment of oxidative stress (Section 1.3.1). The induction of HO-1 is a general response to oxidant stress in mammalian cells (Keyse and Tyrrell, 1989; Applegate *et al.*, 1991) and its altered expression is used as a marker of oxidative stress *in vitro* (Tyrrell and Basu-Modak, 1994). The response of HO-1 to exercise has been examined in a small number of exercise studies, using both animals and humans (Table 1.5).

Table 1.5 Summary of exercise studies investigating HO-1 expression

Reference	n	Exercise	Outcome	Notes
Essig <i>et al.</i> , (1997)	rats n=19 rats n=6	3 h muscular contractions 1 h exhaustive running	↑ HO-1 mRNA 4-fold in muscle 0 h PE ↑ HO-1 mRNA 7-fold in muscle 0 h PE	RT-PCR
Niess <i>et al.</i> , (1999)	n=9 (ET) n=12 (UT)	Half marathon race	↑ leukocyte HO-1 protein 0-24 h PE (ET) ↓ basal leukocyte HO-1 protein in ET compared to UT	Flow cytometry
Niess <i>et al.</i> , (2000)	n=13	Exhaustive run	Neutrophil and monocyte HO-1 protein ↑ PE	Flow cytometry
Pilegaard <i>et al.</i> , (2000)	n=6 n=4	60 min 1LKE repeated for 5 days 4 h cycle at 60% $\dot{V}O_2$ max	Muscle HO-1 mRNA ↑ 11-fold 2 h post 1LKE (day 5) Muscle HO-1 mRNA ↑ 4-fold 0 h PE	RT-PCR
Fehrenbach <i>et al.</i> , (2003)	n=15 n=12 n=12	Exhaustive run 6 x 10 reps eccentric contractions Half marathon race	↑ in neutrophil and lymphocyte HO-1 protein 3 h PE No change in leukocyte HO-1 protein Significant ↑ leukocyte HO-1 protein 0-24 h PE	Flow cytometry
Hildebrandt <i>et al.</i> , (2003)	rats n=5-8	45 min run at 50% $\dot{V}O_2$ max (LIE) 3 h run at 50% $\dot{V}O_2$ max (LIE) 45 min run at 75% $\dot{V}O_2$ max (HIE)	Different muscle types responded differently to exercise. RG ↑ HO-1 mRNA >30 fold 0 h PE (3 h LIE) and WG ↑ HO-1 mRNA >15 fold (3 h LIE/ 45 min HIE)	RT-PCR
Thompson <i>et al.</i> , (2005)	n=8	75 min run at 70% $\dot{V}O_2$ max 75 min rest	Lymphocyte HO-1 mRNA ↑ ~2-fold PE Lymphocyte HO-1 protein ↑ 2 h PE	RT-RTPCR and westerns

PE: post-exercise, ET: endurance-trained, UT: untrained, 1LKE:one-legged knee extensor exercise, RG: red gastrocnemius, WG: white gastrocnemius, HIE: high-intensity exercise, LIE: low-intensity exercise.

The first evidence of increased HO-1 protein expression was reported by Niess and colleagues (1999b) with neutrophil, monocyte and lymphocyte HO-1 protein expression increasing following a half marathon race. The differential expression of HO-1 was associated with the capability of the particular cell type to produce ROS (Thiebault and Mannes, 1997) as the increase in neutrophil and monocyte HO-1 protein was greater than the increase in HO-1 observed in lymphocytes. In addition, the level of IL-8 concentration post-exercise was positively correlated with the level of HO-1 expression in neutrophils (Niess *et al.*, 1999b), supporting a role for HO-1 in the inflammatory response. This investigation observed a difference in the basal expression of HO-1 protein between trained and untrained individuals, with a reduced level of HO-1 found in trained athletes (Niess *et al.*, 1999b). This finding was believed to be the result of training adaptations causing a lower pro-oxidant state through improved antioxidant defences.

Previous work using rats demonstrated that different modes, duration and intensity of exercise affected the induction of HO-1 in skeletal muscle (Essig *et al.*, 1997) with the expression of HO-1 mRNA increasing 7-fold following exhaustive exercise compared to a 4-fold increase immediately after a longer duration of eccentric contractions. The different parameters of exercise and HO-1 mRNA induction in skeletal muscle in humans have been examined, although there are conflicting results. Pilegaard and co-workers (2000) demonstrated a greater expression of HO-1 mRNA in skeletal muscle following a 1 h bout of one-legged knee extensor exercise, performed on five consecutive days, at 70% of their 2 min maximal resistance compared to a prolonged lower intensity cycle. This finding suggests HO-1 induction may be sensitive to intensity, and the fact that HO-1 was induced to a greater extent following one-legged contractions may reflect the increased production of ROS in the muscle with this mode of exercise. The increase in HO-1 expression may be induced in response to exercise to help minimise oxidative damage to skeletal muscle. Furthermore, the induction of HO-1 mRNA in rats in response to exercise of different duration and intensity seems to occur in a fibre type-specific manner (Hildebrandt *et al.*, 2003) suggesting that the induction of HO-1 was required to meet a specific need in the myofibres.

Presently, only one study has examined exercise protocols of differing durations and intensities in human leukocytes. In this study, a large increase in HO-1 protein expression following a half marathon race was observed, with smaller increases evident after an exhaustive run, which was largest in monocytes (Fehrenbach *et al.*, 2003a). In contrast to the evidence provided from studies examining HO-1 in skeletal muscle, no changes in HO-1 protein expression in human leukocytes were observed following

eccentric contractions (Fehrenbach *et al.*, 2003a). Both concentric and eccentric contractions are employed during endurance running, and are more likely to induce damage and produce free radicals than either form of contraction alone (Jackson, 2000). In addition, endurance running has a high mechanical impact and may lead to the destruction of erythrocytes (Jackson, 2000), releasing haem in to the circulation which may be responsible for the increase in HO-1 observed following prolonged running.

Although there is evidence to support an increase in HO-1 protein expression in human leukocytes following exercise, there is limited information concerning the induction of HO-1 mRNA in response to exercise. Thompson and co-workers (2005) provided the first evidence for an accumulation of lymphocyte HO-1 mRNA expression in response to 75 min running at 70% $\dot{V}O_{2max}$. A 2-fold or greater induction of HO-1 mRNA post-exercise was evident in six out of the eight subjects, which was accompanied by increased protein expression 2 h post-exercise. In addition, one subject experienced a remarkable 20-fold increase in HO-1 mRNA 24 h after exercising, indicating a degree of variability in the ability to increase HO-1 expression. This study also found plasma concentrations of IL-6 increased ~ 4-fold immediately post-exercise and it is tempting to speculate that increased lymphocyte HO-1 is a response to oxidative stress initiated by exercise-induced inflammation.

It is apparent that the exercise-induced expression of HO-1 is dependent on the intensity and duration of exercise, with some studies suggesting that intensity is the determining factor (Pilegaard *et al.*, 2000). However, it is likely that a combination of both intensity and duration maximises the HO-1 response. The influence of regular training on HO-1 expression requires further clarification. Although trained individuals demonstrated a lower basal HO-1, they were able to up-regulate HO-1 in response to prolonged exercise (Niess *et al.*, 1999b). In contrast, Pilegaard and colleagues (2000) observed a 2-fold increase in the basal level of HO-1 mRNA expression in skeletal muscle following five consecutive days of one-legged knee extensor exercise in comparison to the non-exercised control leg. Similarly, Boshoff and co-workers (2000), demonstrated an inverse relationship between the inducibility of HSP70 and its basal expression. According to this relationship, a higher basal level of HO-1 would result in a decrease in the HO-1 response to oxidative stress.

In summary, different techniques and exercise protocols have been used to analyse HO-1 with both changes in protein and mRNA reported. Discrepancies in the results may be due to the use of different end points and methods. Collectively, the findings indicate that the induction of HO-1 in skeletal muscle and lymphocytes differs in

response to exercise, possibly implying the up-regulation of HO-1 is dependent on specific requirements of the tissue or that the strength of the stimulus varies between skeletal muscle and lymphocytes.

1.6 Lymphocytes

Lymphocytes play a pivotal role in host defence and the maintenance of normal function. Previous investigations have shown that lymphocytes respond to hyperbaric oxygen treatment (HBO) (Rothfuss *et al.*, 1998), heavy metals (Menzel *et al.*, 1998), heat shock (Rao *et al.*, 1999) and H₂O₂ (Marini *et al.*, 1996a) with an up-regulation of various HSPs and HO-1 following recovery from treatment. The increased expression of these proteins is associated with cellular protection. Since lymphocytes are widely distributed throughout the body, this tissue may provide a clearer picture of the overall situation experienced by the body during oxidative stress. This feature makes lymphocytes an attractive tissue for examining the responses to oxidative stress.

1.6.1 Function and distribution

Lymphocytes are composed of B and T cells, with T cells playing a role in cell-mediated immunity and assisting B cells in antibody production. T cells can be further classified as CD4⁺ cells (T-helper cells) and CD8⁺ cells known as cytotoxic T cells. Another subpopulation of lymphocytes includes NK cells, comprising less than 10% of the total B and T lymphocyte population. As a result of their role in immunity, lymphocytes are distributed throughout the body in tissues and fluids including classical lymphoid organs such as the thymus, lymph nodes and spleen and non-lymphoid organs including the lung, gut and skin (Westermann and Pabst, 1992). In healthy adult humans, it is estimated there are twice as many T cells as B cells with more than 50% of B and T lymphocytes residing in the lymph nodes and spleen and with NK cells located mainly in the liver (35%), lung (25%) and spleen (15%) (Westermann and Pabst, 1992). At any one time, the blood contains very few lymphocytes at only 2% of the total lymphocyte pool. Each day approximately 500×10^9 lymphocytes migrate from the blood in to the lymphoid tissue throughout the body and back to the blood, equivalent to the total lymphocyte number in the body.

Lymphocyte recirculation is a dynamic process involving large numbers of cells moving between a variety of pools (Hay and Andrade, 1998). Lymphocytes continuously migrate from the blood, through tissues into the lymphatic system and lymph nodes, and

back to the blood, providing an important function of immune surveillance of organs and tissues. In addition, lymphocytes are the only leukocyte subpopulation to leave the blood and enter tissues as well as recirculate back to the blood via the lymphatics. Most of the literature investigating lymphocyte recirculation has involved the use of sheep due to the convenient location of the lymph nodes in these animals. The lymphatic vessels draining individual lymph nodes can be accessed surgically in sheep where a single efferent lymphatic drains each lymph node, in contrast to humans where lymph nodes occur in chains (Young, 1999). When a sheep lymph node weighing 1 g received an average 24 ml/h blood, ~25% of lymphocytes entering the lymph node migrated out of the blood through the node into the efferent lymph, reflecting the dynamic process of lymphocyte recirculation (Hay and Hobbs, 1977).

The recirculation of lymphocytes has been monitored in sheep using fluorescent dyes and has shown that the concentration of labelled lymphocytes in lymph is reduced by 50% every 16.5 days (Young and Hay, 1995). During exercise, an immune response is mounted with increases in neutrophils, monocytes and lymphocytes caused by the movement of leukocytes from marginal pools. As the spleen is the largest lymphoid organ it has been suggested that the spleen contributes to changes in lymphocyte number, although studies investigating the effects of splenectomy on lymphocytosis during maximal exercise have provided conflicting results (Iversen *et al.*, 1994; Nielsen *et al.*, 1997). Physical exercise has been shown to affect the distribution and function of NK cells (Shephard and Shek, 1999) as well as the composition of B, T and CD4⁺ and CD8⁺ cells, which increase in concentration during exercise (Nielsen, 2003). As the proportion of CD8⁺ cells has been reported to increase more than CD4⁺ cells, the ratio of CD4/CD8 cells may decline (Nielsen, 2003). During recovery from exercise, lymphocyte cell number declines below resting levels (Field *et al.*, 1991) and this is associated with a decrease in the NK cell number and the proportion of B and T cells.

In summary, lymphocytes represent an appropriate tissue for examining oxidative stress as their ability to recirculate throughout the body may provide more information regarding the overall response to oxidative stress. The blood is a convenient pool to sample from and lymphocytes can be obtained relatively easily. Furthermore, once harvested, the lymphocyte response to oxidative stress can be manipulated *ex vivo* and examined using a range of biomarkers. Therefore, lymphocytes are an appropriate target tissue which can be used to assess the response to oxidative stress.

1.6.2 Lymphocytes and induction of HO-1

The induction of HO-1 in lymphocytes *in vitro* and *ex vivo* has been reported following treatment with different types of oxidant (Applegate *et al.*, 1991; Marini *et al.*, 1996a; Menzel *et al.*, 1998; Speit *et al.*, 2000). Cultured lymphoblastoid cells (TK6) treated with 100 μM H_2O_2 for 30 min resulted in an 8-fold increase in HO-1 mRNA expression between 4 and 8 h of incubation following treatment (Applegate *et al.*, 1991). When lymphocytes were isolated from humans and treated with H_2O_2 (50 μM , 60 min) the induction of HO-1 protein occurred 6 h following treatment and was maintained a further 12 h (Marini *et al.*, 1996a). However, there was a large degree of inter-individual variability in the HO-1 response and this has also been observed in another investigation following treatment with heavy metals (Menzel *et al.*, 1998).

The exogenous administration of H_2O_2 to lymphocytes provides a physiological model of oxidative stress, as this oxidant is generated from nearly all sources of free radical production. In addition, H_2O_2 can diffuse freely in and out of cells and tissues with plasma concentrations of H_2O_2 reported to be $\sim 4\text{--}5$ μM (Yamamoto *et al.*, 1987). Hydrogen peroxide treatment causes DNA damage to lymphocytes (Gardner *et al.*, 1997; Rothfuss *et al.*, 1998; Barbouti *et al.*, 2002). Preconditioning of lymphocytes with an inducer treatment up-regulating HO-1 was associated with reduced DNA damage when cells were challenged with H_2O_2 (Speit *et al.*, 2000). In theory, such an adaptive response following exercise may be of benefit, with HO-1 induction providing protection to lymphocytes against secondary sources of exercise-induced oxidative stress, such as the inflammatory response.

As described earlier (Section 1.5.2), HO-1 induction in lymphocytes provides resistance against apoptosis. Cultured lymphocyte cell lines are routinely used in conjunction with H_2O_2 treatment to investigate oxidative stress and the effects of this oxidant on cell death are well characterised. Although these cell lines provide useful models, the treatment of freshly harvested lymphocytes *ex vivo* may be a more useful model since these cells have not adapted to the conditions of cell culture. Halliwell (2003) discusses the issue of oxidative stress in cell culture and highlights that culture conditions increase free radical production and oxidative stress. Therefore, researchers should be cautious when extrapolating data from cell culture work to the *in vivo* situation (Halliwell, 2003). However, few studies have investigated HO-1 up-regulation in human lymphocytes in response to *ex vivo* H_2O_2 treatment and cell death.

1.6.3 Apoptosis and necrosis

The exposure of lymphocytes to low doses ($< 100 \mu\text{M}$) of H_2O_2 treatment causes lymphocytes to die by apoptosis. However, when lymphocytes are challenged with toxic doses of H_2O_2 the outcome is necrosis, indicating that H_2O_2 cytotoxicity is dependent on concentration (Hampton and Orrenius, 1997). In this investigation, treatment of Jurkat cells with $50 \mu\text{M}$ H_2O_2 induced apoptosis in 25% of cells, whereas $500 \mu\text{M}$ H_2O_2 led to the appearance of necrotic cells. Caspase activation was measured in addition to PS exposure with a 10-fold increase in caspase activity observed in cells treated with $50 \mu\text{M}$ H_2O_2 for 6 h. At higher concentrations ($200 \mu\text{M}$ H_2O_2), a decline in caspase activity was observed with the level of activity falling below levels in control cells. Furthermore, the increase in H_2O_2 concentration to levels that did not activate caspases correlated with the switch from apoptotic to necrotic cell death. Cells treated with low concentrations ($50 \mu\text{M}$) of H_2O_2 were impaired in their ability to undergo Fas-mediated apoptosis. In Jurkat cells pre-treated with anti-Fas prior to addition of H_2O_2 30-60 min after the initial trigger, caspase activity is inhibited by 40%. The findings of this study demonstrate a dual regulation of H_2O_2 by triggering caspases and inducing apoptosis, and balancing apoptosis by the inhibition of caspase activity (Hampton and Orrenius, 1997).

The induction of HO-1 confers cyto-protection against oxidative stress and has been shown to inhibit apoptosis in lymphocytes (Choi *et al.*, 2004). Protection against H_2O_2 -induced apoptosis was found to be related to the (GT) $_n$ repeat promoter polymorphism of the HO-1 gene (Hirai *et al.*, 2003). Lymphoblastoid cells possessing long alleles were more susceptible to apoptosis than those cells with short alleles (Hirai *et al.*, 2003). Cells with short alleles demonstrated a greater induction of HO-1 mRNA expression and HO-1 activity in response to H_2O_2 treatment, reducing apoptosis. Therefore, these findings show that a polymorphism of the HO-1 gene is associated with the strength of induction of HO-1, possibly determining susceptibility to oxidative-stress mediated diseases (Hirai *et al.*, 2003).

The induction of HO-1 is described as a molecular switch that protects against acute oxidative and inflammatory insults (Wagener *et al.*, 2003). Studies using knockout mice have shown that fibroblasts taken from mice deficient in HO-1 had decreased levels of survival in comparison to fibroblasts from control animals (Poss and Tonegawa, 1997b), thus demonstrating the cytoprotective role of HO-1. Furthermore, a case of HO-1 deficiency in humans has been reported (Yachie *et al.*, 1999) displaying symptoms of growth retardation, anaemia, iron deposition, and vulnerability to stressful injury. These injuries are similar to those found in HO-1 deficient mice and illustrate the important role that HO-1 plays in cellular protection *in vivo*. The over-expression of HO-1 in rats

exposed to hyperoxia was anti-apoptotic, protecting cells against hyperoxia-induced lung injury (Otterbein *et al.*, 1999). This was supported by *in vitro* studies showing that over-expression of HO-1 reduces apoptosis in murine fibroblasts (Petrache *et al.*, 2000) and Jurkat cells (Choi *et al.*, 2004). In addition, HO-1 induction is implicated in survival of graft transplants (Soares *et al.*, 2001).

Haem oxygenase-1 induction is a general response to oxidative stress (Keyse and Tyrrell, 1989) and it may be possible to modulate its response by altering redox status. These properties support the use of HO-1 as a useful and relevant marker of oxidative stress. The functional anti-apoptotic nature of this response further strengthens the use of this gene as a molecular marker of oxidative stress *in vitro* and *in vivo*.

1.7 Protection against oxidative stress

Sophisticated intra- and extra-cellular antioxidant systems have evolved to avoid oxidative stress. The antioxidant defence system can be manipulated through the induction of endogenous internal systems or through supplementation with exogenous antioxidants. An antioxidant is defined as ‘any substance, that when present at low concentrations compared to those of an oxidisable compound, significantly delays or inhibits oxidation of that substrate’ (Halliwell and Gutteridge, 1999). The antioxidant defence system comprises many substances, including enzymes which catalytically remove free radicals, proteins which minimise pro-oxidant availability, proteins that protect against oxidative damage (HSPs) and low molecular mass agents that scavenge ROS.

The antioxidant defence system provides a protective mechanism to cells and tissues. The composition of antioxidants differs from tissue to tissue and between cell-types, and this is dependent on (i) which ROS are generated, (ii) the source of ROS production and (iii) where the target of damage is measured (Halliwell and Gutteridge, 1999). Therefore, the antioxidant defence system in each tissue is dependent on the level of oxidative stress encountered. The body has a sophisticated defence system which can be further enhanced through the diet. These two types of defences are described separately in the following sections.

1.7.1 Endogenous antioxidants

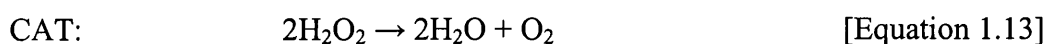
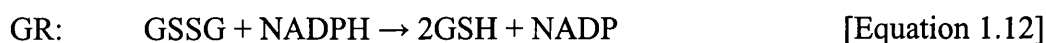
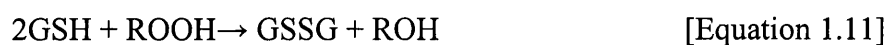
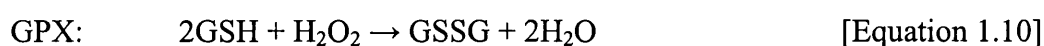
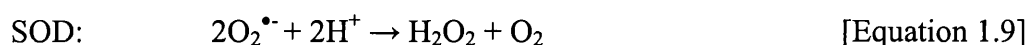
Endogenous protective mechanisms consist of both enzymatic and non-enzymatic antioxidants (Table 1.6), which are located in the intra- and extra-cellular environments offering maximum protection against ROS. The three main antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). These enzymes provide protection through different strategies including the conversion of ROS to less active molecules and the prevention of these less reactive ROS into more deleterious forms of ROS.

Table 1.6 Summary of the major enzymatic and non-enzymatic antioxidants

Antioxidant	Category	Properties
Superoxide dismutase	Enzyme ↓	Dismutates superoxide; Located in the cytosol and mitochondria
Glutathione Peroxidase		Removes hydrogen peroxide and organic hydroperoxides; Located in the cytosol and mitochondria
Catalase		Removes hydrogen peroxide; Located in peroxisomes and mitochondria
Vitamin E	Non-enzymatic ↓	Lipid-soluble; major chain-breaking antioxidant found in cell membranes
Carotenoids		Lipid-soluble; located primarily in tissue membranes
Bilirubin		By-product of haem catabolism; extracellular antioxidant
Ubiquinone		Lipid-soluble quinone derivatives; reduced forms are efficient antioxidants
Flavonoids and polyphenols		Lipid-soluble scavenger and metal chelators
Glutathione		Non-protein thiol in cells
Uric acid		By-product of purine metabolism; scavenges hydroxyl radicals
Vitamin C		Water-soluble; radical scavenger and recycles vitamin E

This is only a short list and does not include all antioxidants. There are many other compounds with protective properties.

The superoxide radical is the precursor to most ROS and is generated at a number of sites. Superoxide dismutase provides the primary cellular defence against superoxide radicals converting it to H_2O_2 and oxygen [Equation 1.9]. Three isozymes of SOD exist, with the CuZnSOD primarily located in the cytosol and the MnSOD found in the mitochondrial matrix (Halliwell and Gutteridge, 1999). Extracellular SOD (EC-SOD) exists in the plasma as well as in tissues. Glutathione peroxidase catalyses the reduction of H_2O_2 to water generating glutathione disulphide (GSSG) [Equation 1.10] or organic hydroperoxides to alcohol and GSSG [Equation 1.11] using reduced glutathione (GSH) as the electron donor. Glutathione peroxidase is a selenium-dependent enzyme and an important cellular protectant against ROS-mediated damage to membrane lipids, proteins and nucleic acids, reducing a variety of hydroperoxides. In order for GPX to function the enzyme requires a supply of GSH and therefore cells must possess a pathway for regeneration of GSH. This occurs via the enzyme glutathione reductase (GR), which uses NADPH to produce a supply of GSH [Equation 1.12]. The distribution of GR is similar to GPX, with greater activity in highly oxidative muscles. Although GR is not a primary antioxidant enzyme, it is essential for the normal antioxidant function of GPX. The function of CAT is to remove H_2O_2 [Equation 1.13], which forms from the dismutation of superoxide. Both CAT and GPX remove H_2O_2 from the environment, however, their affinity for H_2O_2 as a substrate differs with GPX playing a more active role in removing H_2O_2 at low concentrations. Catalase is widely distributed in the cell, with high concentrations in peroxisomes and mitochondria.



The distribution of these enzymes varies from tissue to tissue with highly oxidative skeletal muscle (Type I and IIa fibres) containing a greater enzyme activity of SOD, GPX and CAT than muscles with low oxidative capacity (Powers *et al.*, 1994). Several investigations report an increase in these antioxidant enzymes in skeletal muscle, liver and heart following a single bout of acute exercise-induced oxidative stress in both animal and human studies (Ji and Fu, 1992; Ji *et al.*, 1992; Sen, 1995; McArdle *et al.*,

2001). The effect of regular endurance training has been demonstrated to increase the activity of SOD and GPX along with increased cellular glutathione concentrations in skeletal muscle (Powers *et al.*, 1999). Increased SOD activity was demonstrated in diaphragm muscle of rodents following a 12 week training protocol consisting of 90 min treadmill running at 75% $\dot{V}O_2$ max per day (Vincent *et al.*, 1999). The improved antioxidant capacity resulted in a protective effect against contractile-induced oxidative stress of the diaphragm muscle with significantly lower levels of lipid peroxidation in the trained *versus* sedentary animals (Vincent *et al.*, 1999). This protective effect was also observed following short-term exercise training for five consecutive days (Vincent *et al.*, 2000). Up-regulation of resting SOD and GPX activities has also been demonstrated in human erythrocytes following 12 weeks of high-intensity exercise training (Miyazaki *et al.*, 2001). In this investigation, lipid peroxidation and neutrophil superoxide production in response to incremental cycling to exhaustion were attenuated following the training period, indicating a protective effect of high-intensity exercise training.

The responses to acute and regular exercise are different due to adaptations which occur when exercise is undertaken frequently. Regular exercise training improves the antioxidant defence system and up-regulates the expression of protective proteins lowering the oxidant state of the body (Powers and Sen, 2000). This adaptation ameliorates oxidative stress caused during acute exercise when compared to the degree of oxidative stress induced prior to regular exercise training. However, even in well adapted individuals if the increase in free radical production following exercise is excessive and overwhelms the antioxidant defence system, oxidative stress will occur. Some people may further benefit from antioxidant supplementation to reduce exercise-induced oxidative stress if training adaptations are not sufficient (Sen, 1995; Ashton *et al.*, 1998). As HO-1 is a marker of oxidative stress, an alteration in redox balance will be reflected by changes in the expression of HO-1. In principle, the induction of HO-1 should be modulated by antioxidant availability and confirm both the oxidant-sensitive nature of the gene and the relative importance of any given antioxidant.

1.7.2 Antioxidant compounds

Several non-enzymatic antioxidants are outlined in Table 1.6. Many of these compounds are synthesised *in vivo* or obtained from the diet. Glutathione serves multiple roles in cellular antioxidant defence and is involved in the reduction of vitamin E and vitamin C (Halliwell and Gutteridge, 1999). Bilirubin is a product of haem degradation, catalysed by HO-1, and has antioxidant properties (Stocker *et al.*, 1987). Vitamins A, C, and E,

carotenoids and flavonoids are found in a variety of fruits and vegetables. The localisation of an antioxidant within the body is dependent on its solubility with water-soluble compounds such as vitamin C, GSH and uric acid located in the cytosol, mitochondrial matrix and extracellular fluids. In contrast, the lipid-soluble compounds vitamin A, E, CoQ, carotenoids, polyphenols, and flavonoids are located in membranes, providing protection against oxidative damage to this compartment. A discussion on the protective effects of these antioxidants is beyond the scope of this literature review and the reader is referred to the following articles (Sies and Stahl, 1995; Traber, 2000; Sen, 2001b; Jackson *et al.*, 2002).

In recent years, the purported protective effects of the carotenoid lycopene have attracted attention (Section 1.8). Lycopene is the principal antioxidant in tomatoes and there is strong evidence that tomato consumption is associated with reduced risk of cancer (Giovannucci *et al.*, 1995) and improvement in general health (Gey *et al.*, 1987). In particular, the consumption of a tomato-rich diet has been suggested to be a principle reason for lower cancer rates in the Mediterranean region (La Vecchia, 1998).

1.8 Lycopene

Lycopene is one of over 500 different carotenoids. The predominant feature of carotenoids is the long hydrocarbon chain consisting of a 40 carbon skeleton structure with conjugated double bonds flanked by two β -ionone rings, which provides provitamin A activity (Basu and Dickerson, 1996). This chain is referred to as the polyene chain and is responsible for the photochemical properties and chemical reactivity of carotenoids. Carotenoids are found in all vegetables and fruits containing chlorophyll and are often red, yellow and orange in colour (Britton, 1995). The use of high-performance liquid chromatography (HPLC) analysis has revealed a total of about 34 carotenoids in human serum, including lycopene and an oxidation product of lycopene known as 2,6-cyclolycopene-1,5-diol (Khachik *et al.*, 1997).

1.8.1 Lycopene Structure

Lycopene ($C_{40}H_{56}$) is an acyclic, open chain hydrocarbon containing 11 conjugated and two non-conjugated double bonds arranged in a linear array (Britton, 1995) (Figure 1.6). Lycopene like other carotenoids exists in *cis* and *trans* forms depending on the location of substituent groups, with the *cis*-isomer being generally less stable thermodynamically. The all-*trans* configuration is a linear, rigid molecule that is more

difficult to absorb, solubilise and transport than the *cis*-isomer and for this reason, lycopene is found to exist predominantly as the all-*trans* form in nature. During chemical reactions, the double bonds are capable of undergoing isomerisation from *trans* to mono or poly-*cis* isomers. The most commonly identified forms of lycopene are all-*trans*, 5-*cis*, 9-*cis*, 13-*cis* and 15-*cis*. Lycopene lacks the β -ionone ring structure and consequently provitamin A activity is absent from this molecule. It is now believed that the absence of this structure may in fact increase the antioxidant activity of the molecule (Stahl and Sies, 1996).

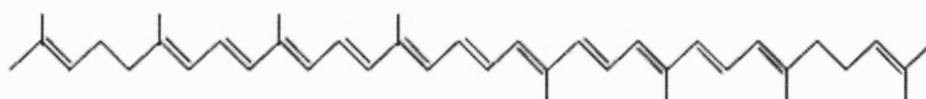


Figure 1.6 Molecular structure of lycopene

The half life of lycopene in human plasma is approximately 2-3 days (Stahl and Sies, 1996). In plasma, the lycopene concentration is approximately 0.3-0.5 μM where it is an isomeric mixture with 50% total lycopene as *cis* isomers (Clinton *et al.*, 1996). Lycopene tends to be found in the all-*trans* form in plant sources thus indicating that *trans* to *cis* isomerisation occurs *in vivo* (Sakomoto *et al.*, 1994), although very little is known about the *in vivo* metabolism of lycopene.

Lycopene is incorporated into dietary lipid micelles and absorbed into the intestinal mucosal lining via passive diffusion. Chylomicrons containing lycopene are released into the lymphatic system for transport to the liver. Lycopene is concentrated in low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) fractions of the serum enabling transportation of lycopene through the membranes to the different organs (Stahl and Sies, 1996). The tissue distribution of dietary carotenoids is not uniform although it is known that lycopene can accumulate in tissues, with the inter-individual variation in tissue lycopene levels about 100-fold (Rao and Agarwal, 1999). Lycopene is most concentrated in the adrenal glands, liver, prostate and testes (Table 1.7), which is believed to be related to the large number of lipoprotein receptors in these tissues.

Table 1.7 Lycopene levels in human tissue

Tissue	Lycopene (nmol/g wet weight)
Testes	4.34-21.36
Adrenal	1.9-21.6
Prostate	0.8
Liver	1.28-5.72
Breast	0.78
Pancreas	0.7
Lung	0.22-0.52
Kidney	0.15-0.62
Colon	0.31
Skin	0.42
Ovary	0.3
Stomach	0.2
Brainstem	Not detectable

(Source: Rao & Agarwal, 1999).

1.8.2 Sources of lycopene and absorption

The most common sources of lycopene are fruits and vegetables that are red in colour, such as tomatoes, watermelon, apricots and papaya (Gross, 1987). When supplementation is via whole foods, it is important to note that the content of lycopene in tomatoes varies with different types of tomatoes and the extent of ripeness (Scott and Hart, 1995). The form in which lycopene is given affects the bioavailability of this antioxidant as *trans-cis* isomerisation occurs during cooking and food processing (Tonucci *et al.*, 1995), therefore there is minimal loss of actual lycopene content. The predominant form of lycopene in raw tomatoes is the all-*trans* isomer, however, the major contributors of lycopene in the Western diet are from tomato juice, ketchup, soup and spaghetti which contain *cis*-isomers (Table 1.8). These foods have one common factor in that they have been processed enabling *trans-cis* isomerisation. Heat treatment

has been found to improve the bioavailability of lycopene causing lycopene release from the food matrix (Stahl and Sies, 1996; Gartner *et al.*, 1997).

Table 1.8 Lycopene contents of common fruits and vegetables

Products	Lycopene ($\mu\text{g/g}$ weight)
Tomato products	
Fresh Tomatoes	8.8-42.0
Cooked Tomatoes	37.0
Tomato Sauce	62.0
Tomato Paste	54.0-1500.00
Tomato Soup (condensed)	79.9
Tomato Powder	1126.3-1264.9
Tomato Juice	50.0-116.0
Pizza Sauce	127.1
Ketchup	99.0-134.0
Other Sources	
Water melon	23.0-72.0
Pink Guava	54.0
Pink Grapefruit	33.6
Papaya	20.0-53.0
Apricot	< 0.1

(Source: Rao and Agarwal, 1998).

The bioavailability of lycopene is enhanced from the food matrix in the presence of dietary lipids, processing and heat-induced isomerisation from all-*trans* to *cis* conformation (Stahl and Sies, 1992). Apart from matrix effects, lycopene absorption from foods is influenced by other factors, namely co-ingestion of high amounts of dietary fibre (Hoffman *et al.*, 1999; Riedl *et al.*, 1999) or by co-ingestion of fat (Clark *et al.*, 2000). Stahl and Sies (1992) demonstrated a 3-fold increase in serum lycopene levels following ingestion of cooked tomato juice in oil medium whereas the

consumption of an equivalent amount of unprocessed tomato juice did not have any effect. Similarly, Gartner and colleagues (1997) found higher levels of lycopene in chylomicron fractions after consumption of tomato paste compared to fresh tomatoes, indicating that the *cis* isomeric form is more bioavailable than the *trans* in tomato paste compared to fresh tomatoes.

1.8.3 Lycopene function

Epidemiological studies examining normal and at risk populations have demonstrated a protective role of lycopene in the prevention of chronic diseases (Table 1.9). A recent review of the epidemiological literature and the relationship between plasma lycopene levels or tomato intake and the reduced risk of cancer has been conducted, finding an inverse relationship in 57 of the 72 studies identified (Giovannucci, 1999). Cancers of the prostate, lung and stomach showed the strongest benefit from lycopene/tomato intake.

Lycopene is a lipid-soluble antioxidant which scavenges both singlet oxygen (Sundquist *et al.*, 1994; Tinkler *et al.*, 1994) and peroxy radicals at a faster rate than any other carotenoid including β -carotene (Di Mascio *et al.*, 1989; Woodall *et al.*, 1997). Additionally, lycopene has been reported to protect lymphocytes from H_2O_2 and nitrogen dioxide radical damage, with protection induced by lycopene at least twice as effective as β -carotene (Bohm *et al.*, 1995). Lycopene has also been found to suppress growth of cancer cells *in vitro* more effectively than other carotenoids (Levy *et al.*, 1995). In this study, a 4-fold and 10-fold relative increase in the concentration of α - and β -carotene was required to produce similar growth suppression of cancer cells as that found with lycopene. The findings from *in vitro* and epidemiological studies provide a strong theoretical relationship between lycopene intake and protection against chronic disease. This has led to an increase in the number of human trials investigating the protective effects of lycopene supplementation.

Table 1.9 The impact of lycopene on chronic diseases in epidemiological studies

Reference	n	Outcome	Notes
Giovannucci <i>et al.</i> , (1995)	773	Dietary tomato-based products >10 vs. 1.5 servings/wk inversely related to prostate cancer risk Tomato sauce intake 2-4 vs. 0 servings/wk inversely related to prostate cancer risk	RR=0.65 RR=0.66
Kohlmeier <i>et al.</i> , (1997)	1379	Inverse OR for MI by quintiles of adipose tissue concentration Contrast between 10 th and 90 th percentile	OR=0.52
Gann <i>et al.</i> , (1999)	578 cases 1294 controls	Plasma LYC strongly associated with lower prostate cancer risk in PLA group	OR=0.40
Slattery <i>et al.</i> , (2000)	2410	Mean intake of LYC 6.4 mg in both cases and controls no difference in LYC and colon cancer risk but inverse relationship with lutein	
Lu <i>et al.</i> , (2001)	65 cases 132 controls	Inverse OR associated with prostate cancer Plasma LYC: 0.223 (cases) vs. 0.307 μ M (controls)	OR=0.17
Polidori <i>et al.</i> , (2001)	72 cases 75 controls	Plasma LYC in controls was 85% greater than diabetics 0.75 \pm 0.25 vs. 0.11 \pm 0.04 μ M	
Giovannucci <i>et al.</i> , (2002)	47 365	LYC intake (>2/week vs. <1/month) associated with \downarrow risk prostate cancer 2481 individuals developed prostate cancer between 1986 and 1998	RR=0.77
Rissanen <i>et al.</i> , (2003)	1028	Serum LYC concentration negatively correlated with mean CCA-IMT	
Erhardt <i>et al.</i> , (2003)	73 cases 63 controls	Median plasma LYC lower in adenoma group than control group (52 μ g.l ⁻¹ vs. 80 μ g.l ⁻¹) Plasma LYC < 70 μ g.l ⁻¹ risk factor for colorectal cancer	

RR: relative risk, OR: odds ratio, MI: myocardial infarction, LYC: lycopene, PLA: placebo, CCA-IMT: common carotid artery-intima media thickness.

1.8.4 Supplementation with lycopene

Several investigations have demonstrated protection to lymphocytes isolated from healthy human subjects supplemented with lycopene through a lycopene isolate or in the form of tomato-based products (Table 1.10). Plasma lycopene concentration increases following supplementation with different tomato products, oleoresin and lycopene beadlets, demonstrating an equal bioavailability of lycopene from these sources (Paetau *et al.*, 1998). A recent study revealed the plasma response of lycopene following consumption of tomato sauce (21 mg/day) for three weeks was 65% greater than the plasma response observed in individuals consuming vegetable juice for three weeks (17 mg/day) (Allen *et al.*, 2003), suggesting that lycopene from tomato sauce is more bioavailable than from vegetable juice. New steady state plasma lycopene concentrations are found within two weeks of increased consumption of tomato products (Micozzi *et al.*, 1992; Allen *et al.*, 2003). Similarly, two weeks consumption of a lycopene free diet has been found to reduce plasma lycopene levels by 50% (Bohm and Bitsch, 1999).

Table 1.10 Lycopene supplementation protects against oxidative stress-induced damage

Reference	n	Supplementation per day	Outcome
Rao and Agarwal (1998)	19	6 treatments, each 1 wk: 2 x SS (20.5/ 39 mg); TJ (50 mg); OLR (75/150 mg); PLA, cross-over	LYC levels ↑ in dose-dependent manner in the SS and OLR ↓ serum TBARS with SS,TJ, OLR
Paetau <i>et al.</i> , (1998)	15	4 treatments each 4 wks: TJ (75 mg); OLR (75 mg); B (70 mg); PLA; each with 4.5 g butter, cross-over	↑ plasma LYC following TJ, OLR and B Detected 2,6-cyclolycopene-1, 5-diol in plasma
Riso <i>et al.</i> , (1999)	10	60 g tomato puree (16.5 mg LYC and 0.6 mg β-carotene) for 3 wks with 10 g olive oil, cross-over	↓ L DNA damage by 33% to 500 μM H ₂ O ₂
Neuman <i>et al.</i> , (2000)	20	OLR (30 mg/day) for 1 wk, cross-over	55% patients protected against exercise-induced asthma Forced expiratory volume in 1 s improved by 10%
Porrini and Riso (2000)	9	25 g tomato puree (7 mg LYC and 0.3 mg β-carotene) for 2 wks with 5 g olive oil, cross-over	↓ L DNA damage by 50% to 500 μM H ₂ O ₂ Inverse relationship between plasma LYC and DNA damage
Stahl <i>et al.</i> , (2001)	19	40 g tomato paste (16 mg LYC) with 10 g olive oil for 10 wks (9 treated, 10 controls)	Dorsal erythema formation was 40% lower in tomato paste group compared to control group
Rao and Shen (2002)	12	6 trials: 5,10, 20 mg LYC of OLR or Ketchup for 2 wks, cross-over	↓ TBARS and protein oxidation with all tomato treatments
Riso <i>et al.</i> , (2004)	20	Tomato products taken with 10 g olive oil for 3 wks (8 mg LYC, 0.5 mg β-carotene, 11 mg vitamin C)	↓ DNA damage by 24% following exposure to iron ions Trend towards ↓ lipid peroxidation in L

SS: spaghetti sauce, TJ: tomato juice, OLR: oleoresin capsules, PLA: placebo, B: lycopene beadlets, LYC: lycopene, L: lymphocytes.

Lycopene supplementation has been shown to increase plasma lycopene and the extent of the increase in tissue lycopene concentration is dependent on the dose of supplementation as well as the form in which lycopene is given (capsule, raw or processed foods). Although extremely high lycopene concentrations can act as a pro-oxidant *in vitro* (Lowe *et al.*, 1999) it seems that in humans it is not physiologically possible to reach these concentrations. Indeed, consumption of up to 150 mg lycopene from a variety of sources (foods and capsules) for one week increased plasma lycopene levels to $\sim 0.75 \mu\text{M}$ (Rao and Agarwal, 1998), revealing that high lycopene concentrations ($> 4 \mu\text{M}$) cannot be achieved by consuming a diet rich in lycopene.

However, this study did demonstrate that one week supplementation of 20-150 mg lycopene per day was sufficient to increase plasma lycopene concentration 2-fold and was associated with reduced endogenous levels of oxidation of lipids, proteins, and DNA. Based on this, recommendations for a daily lycopene intake of 25-30 mg were suggested (Rao and Agarwal, 1998). Supplementation with 25 g tomato puree per day for two weeks increased plasma lycopene levels 3-fold, with a 2-fold increase in the lycopene concentration of lymphocytes (Porrini and Riso, 2000). Both plasma and lymphocyte lycopene concentrations following supplementation were inversely correlated with the level of oxidative DNA damage induced by treatment with H_2O_2 , indicating that lycopene contributes to the protection of DNA from oxidative stress (Porrini and Riso, 2000). The amount of lycopene delivered in this study was 7 mg/day, although more importantly, this dose was delivered in a realistic quantity of a whole food product as previous research has observed protective effects from large quantities of foods or antioxidant isolates (Paetau *et al.*, 1998; Rao and Agarwal, 1998; Paetau *et al.*, 1999; Torbergson and Collins, 2000). This observation was supported by Rao and Shen (2002), demonstrating reduced levels of endogenous lipid peroxidation (TBARS) and protein damage (loss of reduced thiol groups) following two weeks of supplementation with different doses of lycopene (5, 10 and 20 mg/day) in the form of ketchup or oleoresin capsules. In this investigation, there were no differences in the reduction of lipid peroxidation or protein damage between treatments, leading to the conclusion that an intake of 5 to 10 mg lycopene per day is adequate to maintain serum lycopene level and reduce lipid peroxidation. However, this may not be sufficient in populations at high risk for chronic diseases, and the higher recommendation suggested by Rao and Agarwal (1998) may be more beneficial, as epidemiological evidence supports an association between intake of tomato products and the reduced risk of cancer (Giovannucci *et al.*, 1995). Therefore, it is necessary that future investigations continue to examine the effects of lycopene supplementation in food products where the consumption is of manageable and realistic portion sizes.

1.8.5 Antioxidant modulation of HO-1

At the present time, there is no available information on the effect of lycopene supplementation on HO-1 expression in humans and it is only possible to draw parallels with other antioxidants. Generally, the literature indicates a suppressive effect of antioxidant supplementation on the expression of HO-1, and this has been observed in animal models, cell culture studies and in human trials (Table 1.11). For example, rats supplemented with ascorbic acid, bilirubin, β -carotene or α -tocopherol had reduced levels of hepatic HO-1 mRNA accumulation in response to stimulation (Ossola *et al.*, 1997; Yamaguchi *et al.*, 1997).

Presently only two studies have investigated antioxidant supplementation and HO-1 expression in human leukocytes. Niess and co-workers (2000) supplemented athletes with vitamin E for eight days and measured HO-1 protein expression before and after an exhaustive run. In the control trial of this investigation, HO-1 protein expression increased a small amount in neutrophils and monocytes. Vitamin E supplementation did not affect HO-1 expression at baseline and a similar increase in neutrophil and monocyte HO-1 protein was demonstrated following exercise (Niess *et al.*, 2000). Therefore, this study demonstrated that exercise-induced HO-1 protein induction was not modulated by vitamin E supplementation. It is possible that the exercise protocol was not demanding enough, as only small changes in HO-1 were observed. In contrast, larger changes in the cytoplasmic expression of HO-1 have been observed following prolonged running (Niess *et al.*, 1999b), and it is likely that this form of exercise model would be more appropriate to investigate the impact of supplementation.

The second human study examined the effect of a mixed dietary supplement taken daily for five weeks on the expression of HSPs and HO-1 induction following exposure to the free radical generator, AAPH (2, 2'-Azobis-(2-amidinopropane)-dihydrochloride) (Peng *et al.*, 2000). The expression of HSPs and HO-1 were differentially modulated with HSP synthesis increasing and HO-1 protein decreasing at the end of the supplementation period. The overall antioxidant capacity of erythrocytes increased leading to an attenuation of oxidative stress observed by reduced levels of MDA and protein carbonyl formation and a down-regulation in the expression of HO-1. The authors suggested that the reduced HO-1 response may be the result of inhibition of NF- κ B activation as this transcription factor has been found to be inhibited by a number of antioxidants (Sen and Packer, 1996; Jackson *et al.*, 1998).

Table 1.11 Antioxidant treatment and HO-1 expression

Reference	n	Treatment	Outcome
Yamaguchi <i>et al.</i> , (1997)#	66	2 groups (n=33), fed basal diet + AA, or basal diet Rats were deficient in AA synthesis	↓ hepatic HO-1 mRNA expression in AA gp 5 h after LPS stimulation (7-fold vs. 13-fold, respectively)
Ossola <i>et al.</i> , (1997)#	9	Intraperitoneal administration of BR, α-tocopheral or BC 2 h prior to CuSO ₄ treatment in rats	Hepatic HO activity ↓ significantly with BR and α-tocopheral, BC partially ↓ hepatic HO activity
Offord <i>et al.</i> , (2002)*	2	GT-4 fibroblasts treated with 0.1-0.5 μM BC/LYC with 14 μM vitamin C	↑ HO-1 mRNA expression with BC/LYC following 500 kJ.m ⁻² UVA
Trekli <i>et al.</i> , (2003)*	3-4	FEK4 fibroblasts treated with 2.3-21 μM BC	Concentration-dependent ↓ HO-1 mRNA following 250 kJ.m ⁻² UVA with BC (2-fold vs. 6-fold at 2.3 μM)
Basu-Modak <i>et al.</i> , (2003)*	3	FEK4 fibroblasts treated with 30 μM epicatechin	No effect on HO-1 mRNA at 50, 100, 250 kJ.m ⁻² UVA ↑ HO-1 mRNA at 500 kJm ⁻² UVA (12-fold vs.3-fold, respectively)
Niess <i>et al.</i> , (2000)†	13	Vitamin E (500 IU/day) for 8 days	No difference in HO-1 protein expression before or after EE
Peng <i>et al.</i> , (2000)†	32	Mixed antioxidant supplement (1 capsule/day for 5 wks) 19 supplemented and 13 controls	Exposure to AAPH ↓ MDA and protein carbonyl formation ↑ synthesis of HSPs and ↓ HO-1 protein

denotes animal study, * denotes *in vitro* study, † denotes human study, AA: ascorbic acid, AAPH: 2, 2'-Azobis-(2-amidinopropane)-dihydrochloride.

BR: bilirubin, CuSO₄: copper sulphate, BC: β-carotene, EE: exhaustive exercise, LYC: lycopene, UVA: ultraviolet A irradiation.

In contrast to these findings, *in vitro* studies have demonstrated inconsistent results. Cell culture studies using fibroblast cell lines have demonstrated an increase in the expression of HO-1 mRNA following UVA irradiation (500 kJ.m^{-2}) when cells were treated with low concentrations of β -carotene or lycopene (Offord *et al.*, 2002). However, Trekli and colleagues (2003) observed a concentration-dependent suppression of UVA- (250 kJ.m^{-2}) induced transcriptional activation of HO-1 in fibroblast cells by β -carotene. Furthermore, fibroblasts incubated with lycopene in the culture medium also demonstrated suppression of UVA-(100 and 250 kJ.m^{-2}) induced HO-1 mRNA accumulation (Trekli, 2003).

The protective effect of tomato consumption is well established in the literature, although the effect of lycopene supplementation on HO-1 expression in humans has not been investigated. Oxidative stress is implicated in the causation and progression of chronic diseases with epidemiological evidence indicating that diets high in fruits and vegetables are associated with a lower risk of disease (Ames *et al.*, 1993). It is now known that diets high in antioxidants have the potential to modulate the expression of oxidant-responsive genes and it is possible that lycopene is effective through a modulation of redox balance and the response to an oxidant challenge.

1.9 Aims

The research outlined in this thesis aimed to examine the impact of lycopene supplementation on the expression of HO-1. The first part of this thesis aimed to develop models of oxidative stress that were sensitive, reproducible and physiologically relevant. The induction of HO-1 mRNA and protein was used as a marker of oxidative stress. Once an acceptable model was developed, the second stage of this work aimed to investigate whether lycopene supplementation modulates the response to oxidative stress.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

All experimental procedures involving subjects took place in the Applied Physiology Laboratory at the University of Bath. Each study received ethics approval from the NHS Local South West Research Ethics Committee (Bath). Treatments to, and analysis of human mononuclear cells (MNCs) were carried out in the Department of Pharmacy and Pharmacology. This Chapter outlines the generic protocols that were used throughout this thesis. Methods which were used once only are described in the relevant Chapter.

In all studies, excluding Chapter 3, subjects were male volunteers (aged 18-40 years). In Chapter 3, three females volunteered to take part in the study. All subjects were provided with an information sheet detailing the requirements of each study. Subjects gave written informed consent to participate and were asked to complete a medical history questionnaire (Appendix 1). Information regarding physical activity levels were also collected (Appendix 2). Subjects taking regular prescribed medication and smokers were excluded from all studies.

2.2 Anthropometry

Height was measured using a fixed stadiometer (Holtain Ltd., UK) and recorded to the nearest cm. Body mass was measured using an Avery balance scale (Weylux, England) to the nearest 0.05 kg. Subjects were required to wear light clothing during this measurement.

Measurements of skinfold thickness was made in triplicate and measured to the nearest 0.1 mm using skinfold calipers (Holtain Ltd., UK). Four different sites were measured: biceps, triceps, subscapular, and suprailiac. Skinfold thickness was reported as the sum of the four sites.

2.3 Maximal oxygen uptake test

Maximal oxygen uptake ($\dot{V}O_{2\max}$) of each subject was determined using a submaximal speed test followed by an incremental incline test to exhaustion on a treadmill ergometer (Woodway, ELG 70 Weiss, Germany). The submaximal running protocol consisted of four steady-state exercise stages, each lasting four min, with speed increasing by one $\text{km}\cdot\text{h}^{-1}$ with each stage. One-minute expired air samples were collected at the end of each stage of exercise using Douglas bags. Measures of heart rate (HR) (Vantage NV, Polar Electro OY, Finland) and rate of perceived exertion (RPE) using a 6-20 scale (Borg, 1973) were taken during the gas collection.

The percentage of oxygen and carbon dioxide (CO_2) in expired air samples was determined using a paramagnetic O_2 analyser (Series 1400, Servomex Ltd., Sussex, UK) and an infrared CO_2 gas analyser (Series 1400, Servomex Ltd., Sussex, UK). Expired air volume was determined using a dry gas meter (Harvard Apparatus, Kent, UK) and corrected to standard temperature and pressure (dry gas). Expired minute ventilation, respiratory exchange ratio (RER), $\dot{V}O_2$ and carbon dioxide output ($\dot{V}CO_2$) were calculated for each expired air sample.

Following adequate recovery subjects completed the maximal incremental test, consisting of three-minute exercise stages at gradients of 3, 6, 8.5, and 11% until volitional exhaustion. The initial speed for this test was determined by linear regression using HR and speed from the submaximal test, so that HR was approximately 160 $\text{beats}\cdot\text{min}^{-1}$. Expired gas samples were collected in the final min of each stage along with HR and RPE. Peak HR was defined as the highest value recorded during this test. The criteria for a valid $\dot{V}O_{2\max}$ were a maximal RER value of 1.10 and/or maximal HR value within 5% of their age-predicted maximum.

In Chapter 3, the relationship between speed and $\dot{V}O_2$ was used to determine the average intensity at which each subject completed the half marathon race. In Chapter 5, the $\dot{V}O_2$ values were used to calculate the running speeds required to obtain 65, 85 and 100% of $\dot{V}O_{2\max}$.

2.4 Dietary Restrictions

Subjects arrived in the laboratory following an overnight fast on each occasion where a blood sample was taken. Participants in each study were required to report their food

and fluid intake over a period of 72 h prior to each trial and were asked to refrain from consuming alcohol during this period. Each subject received a set of weighing scales (Model 3001, Salter, Kent, UK) to accurately fulfill this requirement. In Chapters 3 to 6, weighed food records were analyzed using the software COMP-EAT 4.0 (Nutrition Systems, UK), which is based on food composition tables.

2.5 Chemicals

All reagents were from Sigma-Aldrich (Poole, UK) unless otherwise specified. Routine tissue culture reagents were purchased from Gibco Invitrogen Ltd. (Paisley, UK). Foetal calf serum (FCS) and horse serum (HS) were purchased from PAA laboratories (Somerset, UK). LightCycler DNA Master SYBR Green I, propidium iodide, Annexin V and bovine serum albumin (BSA) standard solution were purchased from Roche Molecular Biochemicals (Lewes, UK). TRIZOL reagent and Superscript first-strand synthesis kit were purchased from Invitrogen Life Technologies (Paisley, UK). The mouse anti-HO-1 monoclonal antibody (Stressgen, Victoria, Canada) was purchased from Bioquote Ltd (York, UK). Ethanol, methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Tetrahydrofuran (THF) and n-Hexane were purchased from BDH (Poole, UK). Phosphate-buffered saline (PBS) was purchased from Oxoid Ltd. (Basingstoke, UK). Hydrogen peroxide (H_2O_2) was purchased from Calbiochem (Poole, UK).

2.6 Blood Sampling

On the morning of a trial, subjects reported to the laboratory following an overnight fast. Subjects remained in a supine position for 15 min prior to blood sampling. Twenty-five ml of whole blood was collected by venepuncture in the ante-cubital vein. In all studies, syringes were used and the blood dispensed into blood collection tubes (Sarstedt Ltd., Leicester, UK) containing ethylenediaminetetra acetic acid (EDTA) as the anti coagulant. Whole blood differential leukocyte counts, haemoglobin and haematocrit data were obtained using an automatic haematology system (SF-3000, Sysmex Ltd., Milton Keynes, UK).

Whole blood was centrifuged (3500 g, 10 min at 5 °C) to obtain plasma, which was dispensed into eppendorf tubes and immediately frozen at -70 °C. The remaining whole

blood was diluted in RPMI media (containing 10% FCS) to its original volume before isolating peripheral blood mononuclear cells (MNCs) (Section 2.6.1).

In Chapter 5, finger prick samples were obtained before exercise and during the exercise trial, every 15 min, using a lancet (Accu-Check Softclix Pro, Roche, Lewes, UK). The first drop of blood was discarded using a tissue and 100 μ l whole blood was collected using a microvette tube (CB300, Sarstedt Ltd., Leicester, UK) lined with EDTA. Samples were immediately analyzed using a lactate analyzer (2300 STAT Plus, YSI Incorporated, Yellow Springs, USA).

2.6.1 Leukocyte Separation

Peripheral blood MNCs were isolated using a one-step centrifugation technique developed by Boyum (1968). Twenty-five ml diluted whole blood, with the plasma compartment removed was carefully overlaid 12.5 ml Lymphoprep (Nycomed, Norway). The suspension was centrifuged for 20 min at 800 g at 20 °C. The MNC layer formed a distinct band at the interface, which was removed and washed twice with PBS by centrifuging (250 g, 10 min at 20 °C). The cell pellet was collected and resuspended in 20 ml PBS. The total leukocyte cell count was obtained using the SF-3000 and the appropriate volume of cell suspension transferred to falcon tubes for analysis.

2.6.2 Analysis of Mononuclear Cells

In Chapters 3-6, approximately 1×10^6 cells were used for cytosolic extract preparation (Section 2.6.3) for the determination of protein concentration (Section 2.6.4). In Chapter 5, 3×10^6 cells were isolated for immunophenotyping and 1×10^6 cells for indirect immunofluorescence measurement (Chapters 4-6). In Chapter 5, the remaining cell suspension was resuspended in RPMI containing 1% FCS and transferred in to a 150 cm³ flask and placed in a humidified atmosphere at 37 °C with 5% CO₂ for 45 min. Following incubation, the flask was tapped and the supernatant, containing the lymphocyte population, transferred into a 50 ml falcon tube. Monocytes adhere to plastic and these were obtained for RNA extraction following lysis in TRIZOL (Section 2.10). The lymphocyte population was counted and centrifuged (170 g, 5 min at room temperature) and lysed in TRIZOL (Section 2.10). In Chapter 3, the remaining suspension was pelleted and immediately lysed in TRIZOL. In Chapter's 4 and 6, the cell suspension was prepared for treatment with H₂O₂ (Section 2.8.1).

2.6.3 Cytosolic extract preparation

Approximately 1×10^6 cells suspended in PBS were centrifuged (200 g, 5min at 4 °C). The supernatant was aspirated and the cell pellet was resuspended in 50 μ l Munroe lysis buffer containing 10 mM Hepes, pH 7.5; 3 mM $MgCl_2$; 40 μ M KCl; 5% Glycerol and H_2O and 10% NP40, 100 x protease inhibitors and pefabloc. The cell extract was centrifuged (200 g, 7 min at 4 °C) and the supernatant flash-frozen using ethanol and dry ice. This was transferred into 10 μ l aliquots and stored at -70 °C until analysis of protein concentration.

2.6.4 Protein Measurement

Protein content of the samples was determined as described by Bradford (1976). Briefly, BSA standard solution (100 mg/ml) was diluted 1:100 in water and used at concentrations of 0, 2.5, 5, 7, 10, and 15 μ g/ml. For the samples, 5 μ l of the cell extract was transferred into an eppendorf containing 795 μ l water. All samples and standards were vortexed to ensure thorough mixing of the Bradford reagent (BioRad, Hemel Hempstead, UK). The absorbance of 100 μ l aliquots of all standards and samples was measured in triplicate at 550 nm using a microplate reader (MR5000, Dynatech Laboratories, West Sussex, UK).

2.7 Mammalian cell culture

TK6 cells, a B cell line, transfected with Epstein-Barr virus were initially used as they had been shown to over-express HO-1 mRNA (Applegate *et al.*, 1991). These cells were cultured routinely at 37 °C in 5% CO_2 in RPMI-1640 medium, supplemented with 50 u/ml penicillin, 50 μ g/ml streptomycin, 0.2% sodium bicarbonate, and 10% horse serum. These cells were seeded at a density of 5×10^4 cells/ml in 75 cm^3 flasks, and harvested at day two of growth. The media was reserved and kept at 37 °C for use in *ex vivo* treatments. Conditioned media was used for the treatment in all experiments as addition of fresh medium instead of conditioned medium induces HO-1 providing incorrect estimates for basal levels of HO-1 mRNA (Tyrrell and Basu-Modak, 1994).

2.8 Treatment of mononuclear cells

In Chapters 4-6, cells were treated in PBS in 15 ml falcon tubes at a density of 1×10^6 cells/ml, based on cell viability assessed by the MTT assay following incubation in serum free media (SFM) or PBS immediately following treatment (Figure A.1, Appendix 4).

2.8.1 Hydrogen Peroxide Treatment

Mononuclear cell's ($\sim 3 \times 10^6$ cells) were treated in 15 ml centrifuge tubes. Hydrogen peroxide was diluted in PBS to obtain a stock solution of 25 mM H_2O_2 . In Chapter 6, stock solutions of 25 and 45 mM H_2O_2 were used so that exposure was not altered by the volume of solution added. In all cases, isolated human MNCs were exposed to a final concentration of 50 μM H_2O_2 for 30 min at 37 °C. Following treatment, cells were washed once in PBS and then resuspended in conditioned media taken from TK6 cells, and replaced at 37 °C for 4, 6, 18, 24 or 48 h recovery. Following recovery, the cells were centrifuged (170 g, 5 min at room temperature) and placed in aliquots for analysis.

2.8.2 Cell Viability

In Chapters 4 and 5, cell viability was assessed using trypan blue staining. Briefly, 50 μl of sample was diluted 1:2 with PBS, and then 100 μl trypan blue was added. Trypan blue staining is based on the ability of cells with an intact membrane to exclude the dye, therefore it only assesses between live and dead cells and not the mode of death. The ratio of unstained cells to total cell count was calculated and represents cell viability. In Chapter 6, cell viability was assessed by flow cytometry. The effect of H_2O_2 on cell viability was assessed at concentrations of 5, 10, 25, and 50 μM (Figure A.3, Appendix 7) in addition to the effect of the recovery time following treatment (Figure A.4, Appendix 8).

2.9 Flow Cytometry-Indirect Immunofluorescence of HO-1

This technique allows quantification of cells expressing HO-1 protein on a single cell basis within a cell population. In Chapters 4-6, MNCs were analyzed by indirect immunofluorescence using a monoclonal mouse anti-HO-1 antibody IgG1. Following recovery from treatment, $\sim 2 \times 10^6$ cells were centrifuged and fixed and permeabilised in 2 ml 70% ice-cold ethanol. Samples were kept at 4 °C until subsequent analysis. Cells

were washed twice in PBS and then blocked using 500 μ l of a 2% BSA solution for 10 min at 37 °C. Cells were centrifuged and the supernatant discarded. The HO-1 antibody (1:3 dilution used in chapter 4 and 5; 1: 100 dilution in chapter 6) was added directly to the cells and incubated in the dark on ice for 60 min. The cells were washed twice and then incubated in the dark at 4 °C for 45 min in the presence of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse polyvalent immunoglobulins antibody. An isotype control was performed with FITC mouse IgG1 antibody (DAKO, Glostrup, Denmark) and the same anti-mouse IgG-FITC secondary to determine background fluorescence. Cells were washed a further two times in PBS and resuspended in 300 μ l PBS for analysis.

Cell analysis was performed in a Becton Dickinson FACScan (Cellquest version 3.3 software, Belgium) calibration checked with Fluoresbrite beads (Polysciences, Pennsylvania, USA). Samples were analyzed using 488 nm excitation and detection in the green fluorescence channel (FL1-530 \pm 30 nm Bandpass filter). The lymphocyte and monocyte populations were differentiated by size and granularity in the forward *versus* side scatter gram and gated. Background dead cells/debris was excluded by electronic threshold and data for 10,000 events of the lymphocyte population acquired. HO-1 protein was expressed as the fold change in median fluorescence intensity (MFI) of the treated *versus* sham-treated controls. In exercise trials, the MFI of the post-exercise samples was normalised relative to the baseline pre-exercise sample. Percent positive cells (%) were also ascertained to determine similarities between trials.

2.10 Total RNA Preparation

For each sample, one ml TRIZOL reagent per 5×10^6 cells was dispensed into a 15 ml falcon tube and the cells were lysed by pipetting. The monocytes formed a cell monolayer in the culture flasks therefore lysis was performed by adding TRIZOL to the flasks. The lymphocyte and monocyte populations were stored in separate tubes at -70 °C until analysis.

Cells were thawed rapidly at 37 °C and glycogen was added to the lysates at a final concentration of 50 μ g.ml⁻¹ in order to increase RNA yields. Total cellular RNA was extracted according to the manufacturer's protocol (Invitrogen Life Technologies). Briefly, following centrifugation with chloroform, the aqueous phase containing the RNA was extracted and transferred to an eppendorf tube containing isopropyl alcohol. The RNA was recovered by sequential precipitation with 75% cold-ethanol and re-

suspended in RNase free water. The optical density of the isolated RNA was read on a spectrophotometer with an $A_{260/280}$ ratio of 1.6-1.8. This equation is performed as:

$$(A_{260} \times \text{dilution factor} \times 40) / 1000 = \text{RNA concentration } (\mu\text{g} \cdot \text{ml}^{-1})$$

2.10.1 Reverse-Transcription-PCR (RT-PCR)

A two-step RT-PCR approach using Superscript first strand synthesis kit was used to analyze HO-1 mRNA accumulation. Random hexamers were used for priming the reaction to obtain a better representation of the RNA population in the cDNA samples. Briefly, an RNA primer mix containing 2 μg total RNA, random hexamers (50 $\text{ng} \cdot \mu\text{l}^{-1}$), 10 mM dNTP mix, and DEPC-treated water was denatured at 70 °C for 5 min and chilled immediately on ice. Reaction components for RT-positive and RT-negative samples were set up separately. Nine μl of the reaction mix (10 x reaction buffer, 25 mM MgCl_2 , 0.1 M DTT, RNaseOUT (40 $\text{U} \cdot \mu\text{l}^{-1}$), was added to each RT sample and incubated at room temperature for 2 min. Superscript II (50 $\text{U} \cdot \mu\text{l}^{-1}$) was added to the positive RT samples and incubated for 10 min at room temperature. All samples were incubated at 42 °C for 50 min, then 70 °C for 15 min and immediately chilled on ice. RNase H (2 $\text{U} \cdot \mu\text{l}^{-1}$) was added to each positive RT sample and incubated at 37 °C for 20 min. The newly synthesized cDNA was stored at -20 °C until required for real-time RT-PCR.

2.10.2 Real-Time RT-PCR

Real-Time RT-PCR was used for quantification of HO-1 mRNA in human mononuclear cells in both exercise studies (Chapters 3 and 5). Reactions were carried out in the LightCycler (Roche Molecular Biochemicals, Lewes, UK) using the fluorescent green dye SYBR Green I. The light cycler performs a quantification analysis by comparing the fluorescence of a PCR product of unknown concentration with the fluorescence of several dilutions of an external standard. SYBR Green I dye binds to all double-stranded DNA molecules regardless of their sequence and produces fluorescence proportional to the amount of amplified DNA. Fluorescence is acquired once per cycle generating a curve that increases in value with each cycle as the product accumulates (Figure 2.1). The use of relative quantification (RelQuant software, Roche Diagnostics, Lewes, UK) allows the result to be expressed as a relative ratio of the target of interest (HO-1) to a reference target (GAPDH) measured in the same sample material. This method has been described by Basu-Modak and colleagues (2003).

Briefly, a 1:10 dilution of each cDNA was freshly prepared in PCR grade water before each run and 2 μ l was used in each PCR reaction. The total volume for PCR reactions was 20 μ l. This reaction included 3 mM $MgCl_2$ in PCR grade water, 2 μ l of SYBR Green I dye and primer pairs at a final concentration of 0.5 μ M. The experimental protocol consisted of four programs including predenaturation, amplification, melting curve analysis and cooling (LightCycler 3 Run version 5.32). The temperature transition rate of 20 $^{\circ}C.s^{-1}$ was used in all programs unless specified.

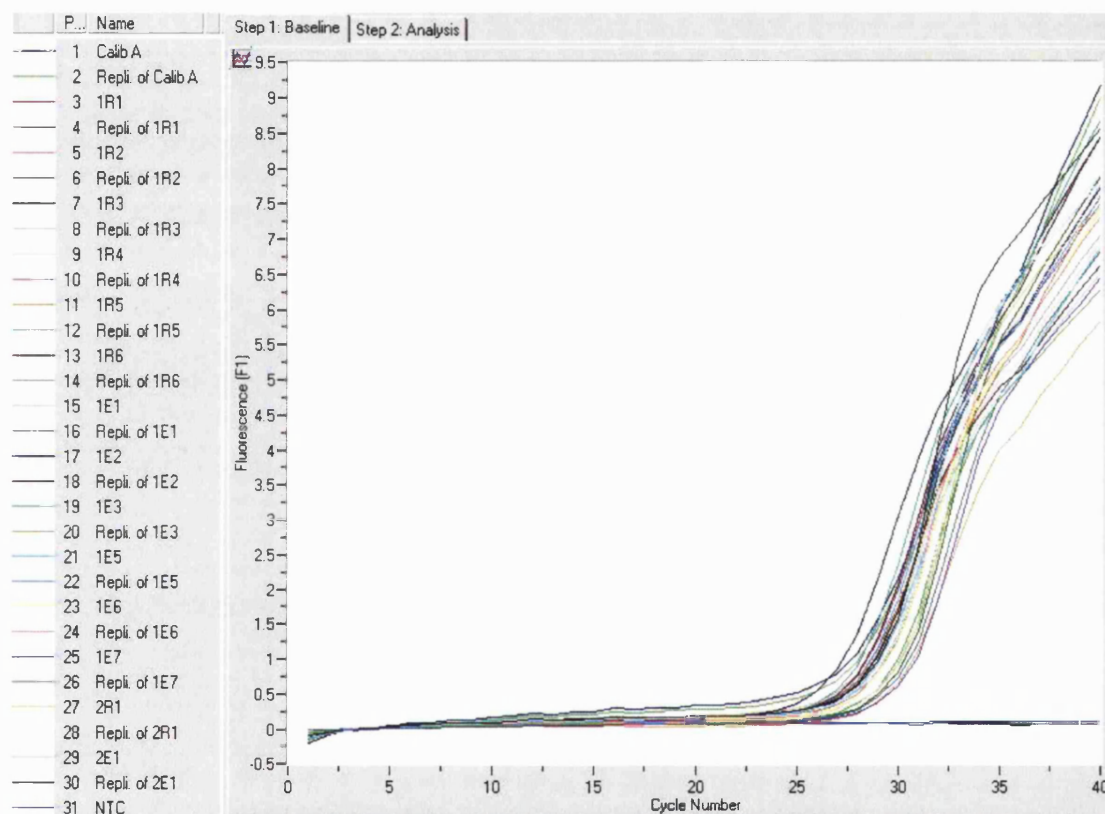


Figure 2.1 Representation of the LightCycler software analysis package. The software calculates the increase in fluorescence of SYBR Green I dye with increasing cycle number. Each sample was analysed in duplicate, with a calibrator and a no template control (NTC). Each colour represents a single sample. R: rest sample, E: exercise sample.

The primer sets had different amplicon sizes therefore the optimal amplification conditions were slightly different, and this meant that the gene of interest and the reference gene were analysed in separate runs (Figure 2.2). The parameters for HO-1 (target) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference) are outlined in Table 2.1.

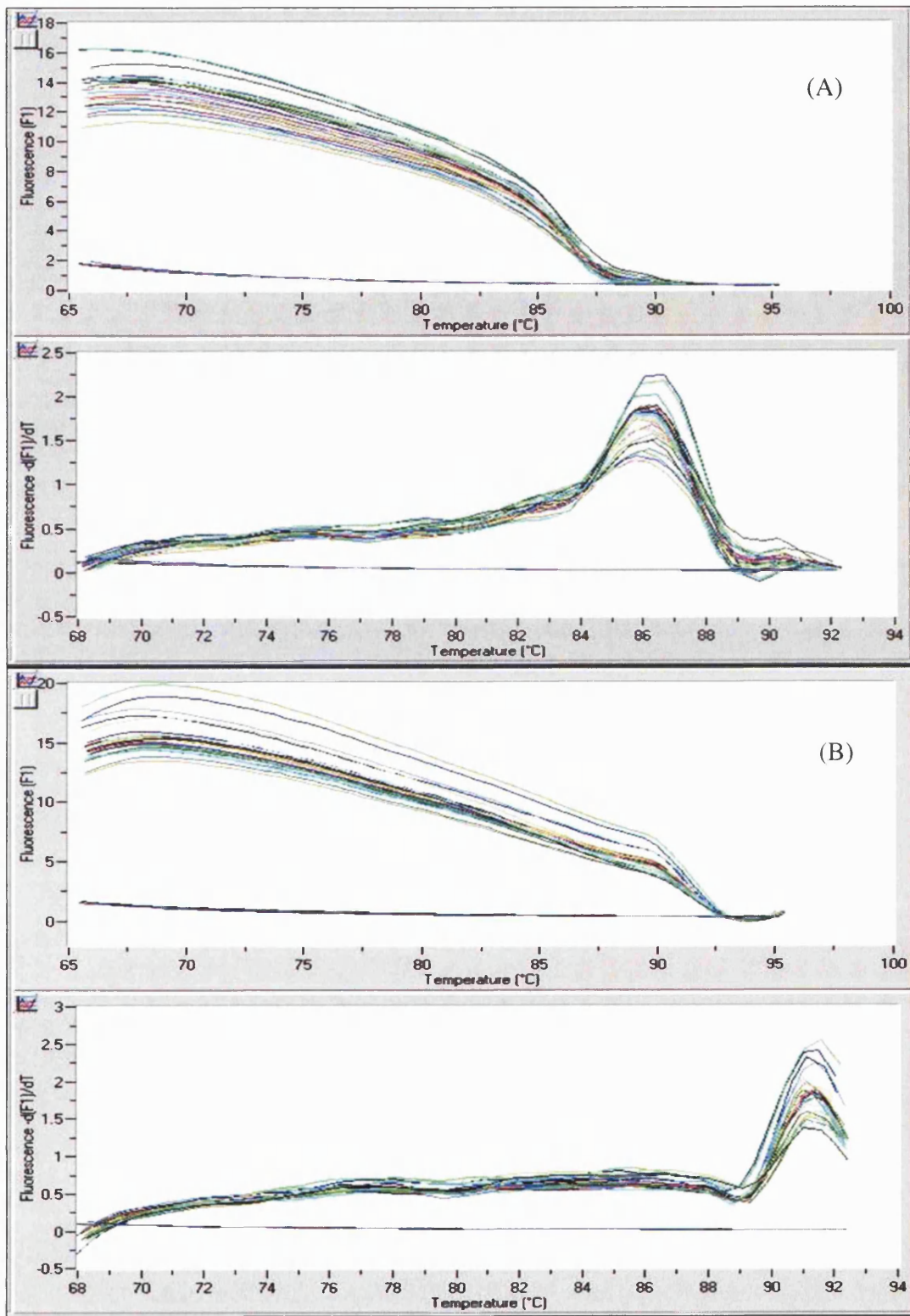


Figure 2.2 Melting curve analysis for HO-1 (A) and GAPDH (B) accounting for the differences in run conditions.

Table 2.1 Experimental protocol used for the different stages of Real-Time RT-PCR

	HO-1	GAPDH
Predenaturation	1 cycle of 30 s at 95 °C	
Amplification	40 cycles of: 0 s at 95 °C 5 s at 55 °C 4 s at 72 °C	40 cycles of: 0 s at 95 °C 5 s at 55 °C 8 s at 72 °C
Melting Curve Analysis	1 cycle of: 0 s at 95 °C 15 s at 65 °C Gradual ↑ to 95 °C (transition rate of 0.1 °C) with continuous fluorescence acquisition	
Cooling	Default parameters	

The HO-1 primer pair 5'AAG AGG CCA AGA CTG TC-3' (forward) and 5'-GGT GTC ATG GGT CAG CAG C-3' (reverse) gave an amplicon size of 74 bp in the human HO-1 cDNA sequence. The GAPDH primer pair 5'-GAC ATC AAG AAG GTG GTG AA-3' (forward) and 5'-TGT CAT ACC AGG AAA TGA GC-3' (reverse) had an amplicon size of 178 bp in the human GAPDH cDNA sequence. Desalted primers were obtained from Life Technologies (Paisley, UK) and used for PCR reactions without further purification.

An external standard curve was developed using serial dilutions in the range of 1:10 to 1:3000 of a cDNA pool obtained following recovery from irradiation with 250 kJ.m⁻² UVA. The concentration of cDNA in this pool was used to generate separate standard curves for HO-1 and GAPDH. Triplicates of each dilution were used for the standard curve runs and the data from these runs were used to create a coefficient file in the Relative Quantification software.

A calibrator was used in all light cycler runs and consisted of cDNA with a relatively high HO-1 signal. This was diluted 1:10 in PCR grade water and run in duplicate for each set of sample runs. RelQuant software was used to analyze the data file of each run. These files were exported and the ratio concentrations were determined using the dual colour mode with efficiency correction. The normalised HO-1 mRNA values in the post-exercise samples were expressed as a fold increase of the pre-exercise, control sample. The reproducibility of the reverse-transcriptions were assessed in addition to the reproducibility of the light cycler runs, by repeating three RT's and running the cDNA samples in the light cycler (Figure A.2 (A), Appendix 5) and by running the same cDNA sample in the light cycler for three separate runs for HO-1 and GAPDH (Figure A.2 (B), Appendix 5).

2.11 Cytokines

Plasma interleukin-6 (IL-6) and interleukin-10 (IL-10) were determined in some Chapter's using a commercially available solid phase enzyme linked immunosorbent assay (ELISA). In Chapter 3, a high sensitivity kit for IL-6 was used (Quantikine, R and D systems Inc., Abingdon, UK) with the minimum detectable concentration claimed by the manufacturer to be 0.016 to 0.110 pg.ml⁻¹. In Chapter 5, plasma IL-6 and IL-10 concentrations were analysed using high sensitivity kits (Quantikine, R and D systems Inc., Abingdon, UK) with the minimum detectable concentration for IL-10 claimed to be less than 0.5 pg.ml⁻¹. Samples were analysed in duplicate in a microplate reader and read at 450 nm with wavelength correction at 690 nm (Anthos HT-III, Labtech International Ltd., Ringmer, East Sussex).

CHAPTER 3

HO-1 mRNA EXPRESSION IN HUMAN MONONUCLEAR CELLS FOLLOWING A HALF MARATHON RACE

3.1 Introduction

There is a wealth of evidence relating the benefits of regular exercise to health, with associations of increased risk to disease and decreased longevity with sedentary behaviour. Although the rewards of regular exercise greatly outweigh the costs, it is widely accepted that an acute bout of demanding or non-familiar exercise leads to oxidative stress (Jackson, 2000). The induction of the antioxidant enzyme haem oxygenase-1 (HO-1) is a general response to oxidant stress in mammalian cells (Keyse and Tyrrell, 1989) and has been shown to be up-regulated in response to a wide variety of inducers (Morse and Choi, 2002). Haem oxygenase-1 is an appropriate gene to monitor oxidative stress (Offord *et al.*, 2000) although, for it to be validated as a marker of oxidative stress *in vivo*, the response needs to be characterised further.

The first evidence reporting an induction of HO-1 in leukocytes following exercise was by Niess and co-workers (1999b). In this investigation, flow cytometric analysis of leukocytes revealed an elevation in HO-1 protein expression immediately after a competitive half marathon. In a later study by the same group, HO-1 protein expression was greater following a half marathon in comparison to a short exhaustive run and an eccentric exercise protocol (Fehrenbach *et al.*, 2003a), supporting the use of prolonged endurance exercise in the investigation of exercise-induced HO-1. An increase in human lymphocyte HO-1 mRNA following exercise has only recently been reported (Thompson *et al.*, 2005). In this study, the induction of HO-1 mRNA was accompanied by an increase in HO-1 protein 2 h following prolonged exercise at 70% $\dot{V}O_{2\max}$ for 75 min. The induction of HO-1 has been shown to be cytoprotective (Vile *et al.*, 1994) as well as anti-inflammatory (Willis *et al.*, 1996) and, as acute exercise causes both oxidative stress and inflammation *in vivo*, the induction of HO-1 may have important implications for leukocyte function.

Although a half marathon race is assumed to be more physically demanding than the prolonged run used by Thompson and colleagues (2005), the intensity of exercise was not assessed by Niess and co-workers (1999b) and it is possible that the induction of

HO-1 mRNA in response to a half marathon is greater than the level observed by Thompson and co-workers (2005) following running for 75 min at 70% $\dot{V}O_{2\max}$. Both protocols have shown HO-1 protein induction, and if HO-1 mRNA expression is higher in response to a half marathon, then this type of exercise may be a useful model of exercise-induced oxidative stress. Therefore, the aim of the present investigation was to examine changes in HO-1 expression, at both the mRNA and protein level, in response to a competitive half marathon race.

3.2 Methods

3.2.1 Subjects

Nine students, six males and three females, volunteered to participate in this study (Table 3.1). All participants had entered the Bath half marathon race and had trained, for this event in particular, for at least three months prior to the race. Four individuals had never attempted the distance. Physical activity was assessed by questionnaire (Appendix 2). All subjects reported to the laboratory for preliminary measurements to be taken two weeks prior to the race.

Table 3.1 Subject characteristics

Age (yrs)	Height (cm)	Mass (kg)	$\dot{V}O_{2\max}$ (ml.kg ⁻¹ .min ⁻¹)	Σ 4 Skinfolds (mm)	Weekly exercise (h)
24.1 \pm 1.6	170 \pm 10	64.2 \pm 7.2	61 \pm 7	26 \pm 8	8.0 \pm 7.6

Values are mean \pm S.D. (n=9).

3.2.2 Experimental design and procedures

Subjects kept a record of their food and fluid intake for three days prior to the race (Chapter 2.4) and refrained from strenuous physical activity for at least 48 h before racing. On the day of the competition, subjects arrived at the laboratory following an overnight fast. A resting blood sample was taken by venepuncture from a forearm vein after the subject had been seated for 15 min (2 h prior to the race; Pre-exercise), and then at 1 h, 3 h and 24 h following completion of the race (Figure 3.1). Heart rate was monitored continuously throughout the race by short-range telemetry (Vantage NV, Polar Electro OY, Finland). The time to complete the event was noted to determine estimates of mean speed and exercise intensity.

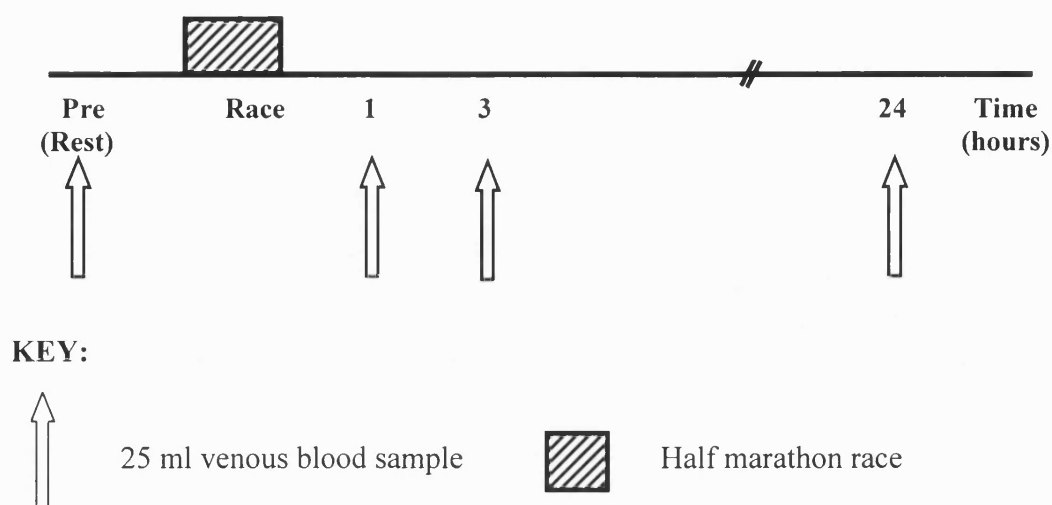


Figure 3.1 Schematic design of the half marathon race day.

Complete blood cell counts including haemoglobin and haematocrit and differential leukocyte counts were assessed using an automated haematology counter (Chapter 2.6). Plasma aliquots were stored at -70°C for subsequent analysis of cytokines (Chapter 2.11). Mononuclear cells were harvested and isolated (Chapter 2.6.1) for the analysis of HO-1 mRNA using real-time RT-PCR (Chapter 2.10) and HO-1 protein expression.

Western blotting was used to determine HO-1 protein induction following protein measurement (Chapter 2.6.4) of the cytosolic extract (Chapter 2.6.3). Equal amounts of protein ($40\text{ }\mu\text{g}$) were diluted with 3 x loading buffer (180 mM Tris, pH 6.8, 3% SDS, 150 mM 1,4-dithio-DL-threitol (DTT), 30% glycerol, 0.0015% bromophenol blue, 0.53 ml milliQ water) and boiled for 5 min. A 12.5% polyacrylamide gel was used for protein separation by electrophoresis at 80 V for 2 h (Mini-protean 3, BioRad, Hemel Hempstead, UK). Samples were transferred onto nitrocellulose membranes for 1 h at 100 V (Trans-blot cell system, BioRad, UK) that were blocked overnight at 4°C in blocking solution (3% milk powder, 0.05% Tween-20 in PBS for HO-1 antibody and with 3% milk powder, 3% BSA, 0.1% Tween-20 in PBS for the actin antibody). After blocking, blots were probed with a monoclonal mouse anti-HO-1 antibody or monoclonal mouse anti-actin antibody (Amersham Biosciences, Bucks, UK) at 1:200 dilution in blocking solution containing 0.05% Tween-PBS for 1 h at room temperature. Following incubation with the primary antibody, blots were washed three times with Tween-PBS. Both proteins were detected with a rabbit anti-mouse IgG-HRP conjugate at a dilution of 1:1000, incubated for 1 h at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence detection system (Amersham

Biosciences, Bucks, UK). HO-1 was normalised to β -actin and expressed relative to baseline samples.

3.2.3 Statistical Analysis

A one-way analysis of variance (ANOVA) with repeated measures was used to analyse the changes in white blood cell populations and both protein and RNA yield and HO-1 mRNA expression across time. The data was not normally distributed therefore a natural log transformation was applied. *Post hoc* analysis using a paired *t* statistic was used to determine at which time points a significant effect was observed. Significance was accepted at the 5% level. Values are expressed as mean \pm standard deviation (S.D.). Data was analysed using SPSS version 10 (SPSS Inc., Chicago, USA).

Effect size statistics (ES) were calculated using the ratio of the mean difference to the grouped standard deviation for the baseline and post-exercise time points for all parameters (Equation 3.1). Small, moderate and large effect sizes were indicated by ratios of 0.2-0.49, 0.5-0.79, and 0.8 upwards, respectively (Cohen, 1988).

$$ES (d) = (M_1 - M_2) / \sigma_{\text{pooled}} \quad \text{where } \sigma_{\text{pooled}} = \sqrt{[(\sigma_1^2 + \sigma_2^2) / 2]} \quad [\text{Equation 3.1}]$$

The relationship between the fold increase in plasma IL-6 concentration and peak HO-1 mRNA expression following the half marathon was determined using Pearson's product moment correlation coefficient (*r*). All the results from the blood analysis for HO-1 are the mean of eight rather than nine subjects, as difficulties were encountered with the extraction procedures for one subject.

3.3 Results

Race Data

Mean HR during the race was $176 \pm 7 \text{ b} \cdot \text{min}^{-1}$ ($n=9$). The time for completion of the race ranged from 80 min to 121 min with the mean time for these individuals being 103 ± 13 min. The mean exercise intensity at which the individuals completed the race was $73 \pm 5\% \dot{V}O_{2\text{max}}$.

Dietary information was collected over a three-day period prior to the race (Table 3.2). The large variation in energy intake is a result of a mixed population, as it is

acknowledged that energy intake is greater in males (3293 ± 660 kcal; $n=6$) compared to females (2035 ± 400 kcal; $n=3$).

Table 3.2 Daily dietary composition assessed 72 h prior to the race

Energy Intake (kcal)	Carbohydrate (%)	Protein (%)	Fat (%)
2874 ± 841	56 ± 6	15 ± 1	29 ± 6

Values are mean \pm S.D. ($n=9$).

Exercise-Induced Leukocyte Cell Changes

The differential leukocyte counts changed following the half marathon (Table 3.3). Neutrophils and monocytes increased significantly at 1 h ($ES = 1.7$, $P = 0.001$ and $ES = 1.5$, $P = 0.001$ in neutrophils and monocytes, respectively) and 3 h following the half marathon ($ES = 1.75$, $P = 0.000$ and $ES = 1.44$, $P = 0.015$ for neutrophils and monocytes, respectively), with the largest increase in the neutrophil population accounting for the significant change in total white blood cell number. In contrast, the number of lymphocytes was lower 1 h ($ES = -1.34$, $P = 0.002$) and 3 h ($ES = -1.14$, $P = 0.048$) following completion of the race. At 24 h, the cell populations tended to be above baseline resting values, although this was not statistically significant.

Table 3.3 Differential leukocyte counts at rest, 1, 3, and 24 h after the half marathon

	White blood cells	Neutrophils	Lymphocytes	Monocytes
Baseline	4.64 ± 0.97	2.47 ± 0.58	1.63 ± 0.41	0.31 ± 0.11
1 h	$15.39 \pm 4.48^{**}$	$13.57 \pm 4.38^{**}$	$1.04 \pm 0.22^{**}$	$0.70 \pm 0.21^{**}$
3 h	$14.28 \pm 3.44^{**}$	$12.47 \pm 3.51^{**}$	$1.18 \pm 0.22^{*}$	$0.57 \pm 0.14^{*}$
24 h	5.87 ± 1.41	3.45 ± 1.31	1.83 ± 0.38	0.40 ± 0.12

Units are cell number ($\times 10^9 \cdot l^{-1}$). Values are means \pm S.D. ** denotes significant changes compared to resting values ($P < 0.01$), * ($P < 0.05$). ($n=8$).

RNA yield

The total RNA yield at 1 h post-race was significantly greater compared to pre-race values (ES = 1.23, $P = 0.005$) (Figure 3.2).

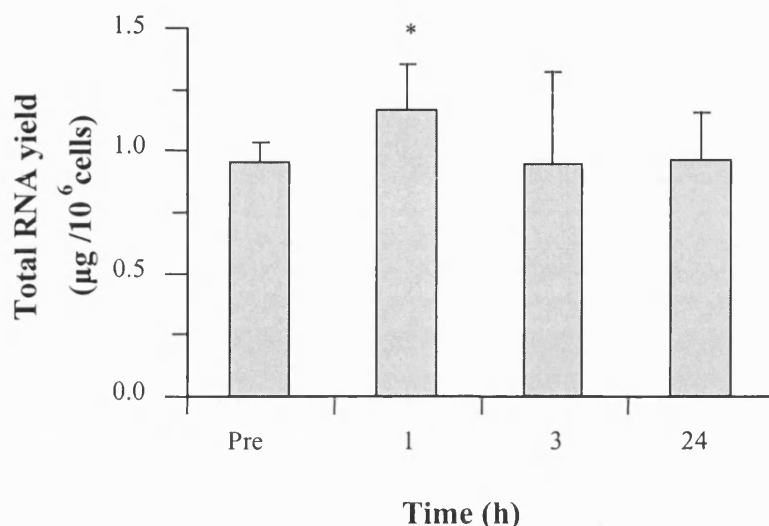


Figure 3.2 RNA yield ($\mu\text{g}/10^6$ cells) in MNCs, at rest before the race and 1, 3, and 24 h following the race. Pre: pre-exercise. * denotes a significant difference from pre-race values ($P < 0.01$). Values are expressed as mean \pm S.D. ($n=8$).

HO-1 mRNA expression

In five of the eight subjects, HO-1 mRNA expression in MNCs changed relative to their respective pre-exercise sample by 1.5-fold at the 1 h and 3 h post-race time points. However, the magnitude of change of HO-1 mRNA induction following the half marathon varied dramatically among the individuals (Figure 3.3), which gave an overall non-significant effect of HO-1 mRNA induction over time ($P = 0.408$). HO-1 mRNA expression was greatest in subject 7 at 3 h post-race and returned to resting values 24 h later. The peak level of HO-1 mRNA expression in subjects 3 and 5 was at 1 h post-race with the level at 3 h falling below resting values. Subject 1 had an increase in HO-1 mRNA at 1 h post-race that had returned to the baseline level by 3 h post-race. Although the mean change in HO-1 mRNA induction was not significant, effect size statistics at 1 and 24 h post-exercise, indicated moderate and large effects, respectively (Table 3.4).

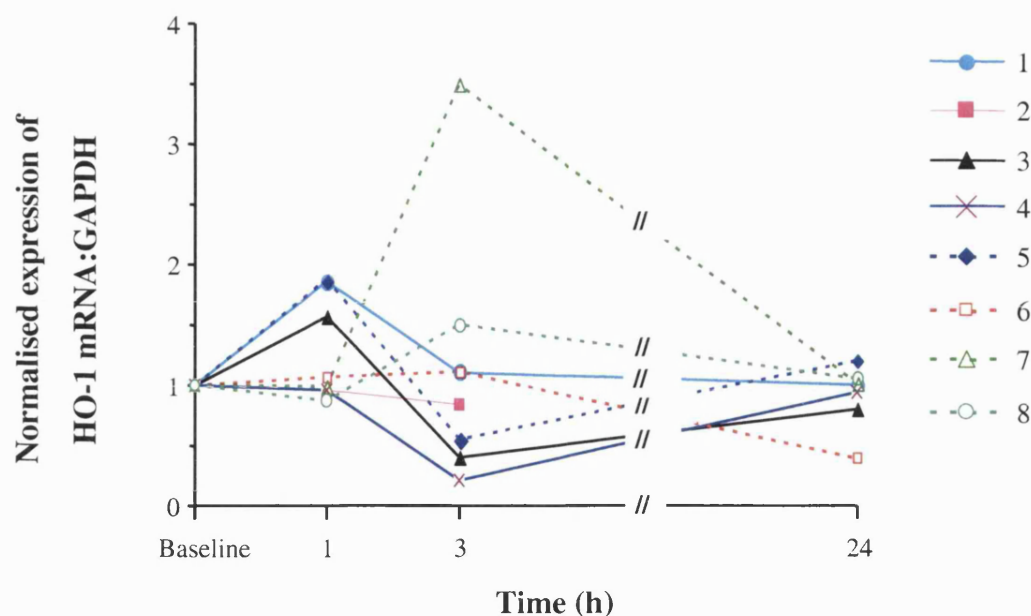


Figure 3.3 HO-1 mRNA expression in human MNCs prior to the half marathon at rest, and 1, 3 and 24 h post-race in eight individuals. HO-1 mRNA is expressed relative to GAPDH. Data is expressed as the fold change compared to each individual baseline (resting) sample. For subject 2, data was unavailable 24 h post-race.

Table 3.4 The fold change in HO-1 mRNA at 1, 3, and 24 h post-exercise and associated effect size statistic

Time post-exercise (h)	Mean	S.D.	ES
1	1.27	0.43	0.74
3	1.15	1.05	0.19
24	0.91	0.26	-1.02

The ES of 0.74 and -1.02 indicate a moderate and large effect at 1 and 24 h post-exercise, respectively. (n=8 at 1 and 3 h post-exercise; n=7 at 24 h post-exercise).

The variability in the MNC induction of HO-1 mRNA is clearly demonstrated in Figure 3.4 showing the peak value observed for each subject against the resting baseline value.

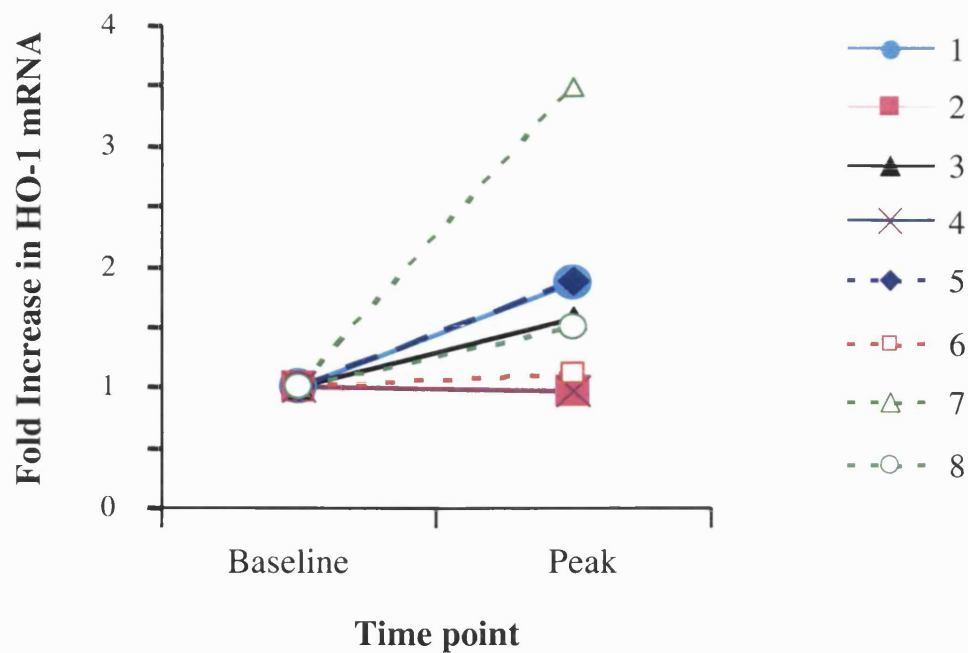


Figure 3.4 Peak exercise-induced fold change in HO-1 mRNA expressed against the baseline level (resting sample). The peak time point was at 1 h post-race for subjects 1, 3 and 5 and at 3 h post-race for subject's 7 and 8.

In order to explore whether variability in the induction of HO-1 was related to basal expression of HO-1, a comparison of each individuals' basal expression and induction of HO-1 mRNA following exercise was performed (Figure 3.5). In addition, the basal expression of HO-1 mRNA in these subjects was also compared to the resting level of HO-1 mRNA in a different group of eight subjects who did not perform the half marathon (Figure 3.6).

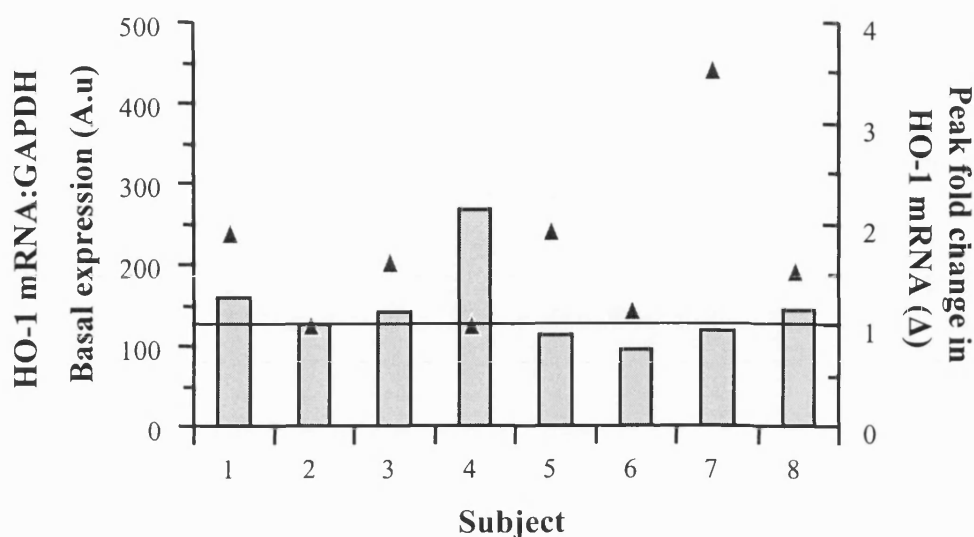


Figure 3.5 Comparison of the basal level and peak fold induction of HO-1 mRNA following a half marathon race. Bars represent the basal expression of HO-1 mRNA with triangles representing the peak fold change. The black line at 1 on the right hand scale indicates the resting value of HO-1 mRNA which is used for normalising the data, therefore solid triangles above this line show increased HO-1 mRNA induction.

It appears that basal expression of HO-1 was not related to the extent of peak induction of HO-1 mRNA following a half marathon ($r = -0.295$, $P = 0.478$).

In Figure 3.6 it is clearly demonstrated that resting levels of HO-1 mRNA in samples 1-8 (taken from subjects before the half marathon), were higher than in samples 10-17 (taken from subjects performing a rest trial). Basal HO-1 mRNA in samples 10-17 were very different to those of 1-8, with the mean HO-1 mRNA expression of samples 1-8 significantly higher than the mean HO-1 mRNA expression of samples 10-17 ($ES = 1.46$, $P = 0.004$). The second group of subjects refrained from exercise and drinking alcohol for two days prior to the trial. These subjects were taking part in a rest trial similar to that described in Chapter 5. For this second group of individuals, mean age, height, body mass and sum of skinfolds were 21 ± 1 yrs, 179 ± 1 cm, 73.9 ± 2.4 kg, 26 ± 2 mm (mean \pm S.D.), respectively. Mean $\dot{V}O_2$ max was 64 ± 2 ml.kg⁻¹.min⁻¹. All subjects in this group were male, therefore, the only difference in these two groups of subjects was gender.

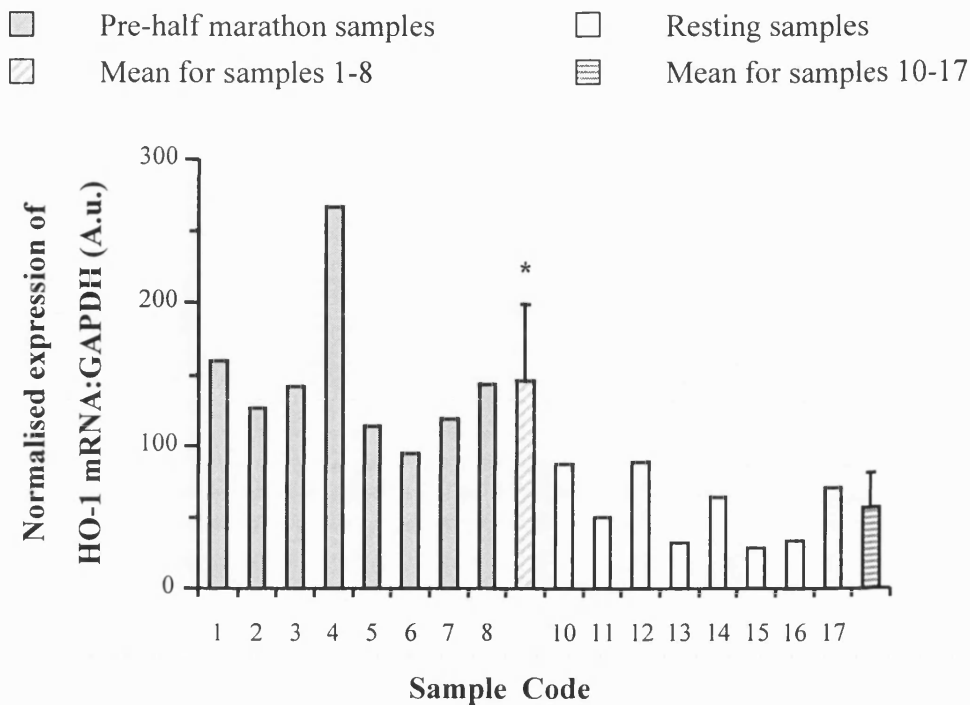


Figure 3.6 Comparison of basal HO-1 mRNA expression in human MNCs in 16 individuals. Each code represents the subject number. Samples 1-8 were taken prior to a half marathon race whereas samples 10-17 were taken from eight different subjects participating in a rest trial. All venous samples were taken in the morning. The means \pm S.D. are also included. * denotes a significant difference between the two groups in the HO-1 mRNA basal levels ($P < 0.01$). Subject characteristics for samples 10-17 are detailed on page 75.

Protein Expression

The protein concentration of MNC extracts varied among the individuals and no significant differences were observed over time ($P = 0.240$). Technical difficulties were encountered when performing western blot analysis of the cell extracts resulting in no available data for HO-1 protein expression.

Cytokines

Plasma interleukin-6 (IL-6) concentration 1 h post-exercise was significantly greater than pre-exercise values (ES = 1.73, $P = 0.000$) (Figure 3.7). A positive relationship between the fold increase in plasma IL-6 concentration and the peak fold increase in HO-1 mRNA expression was observed ($r = 0.776$, $P = 0.024$) (Figure 3.8).

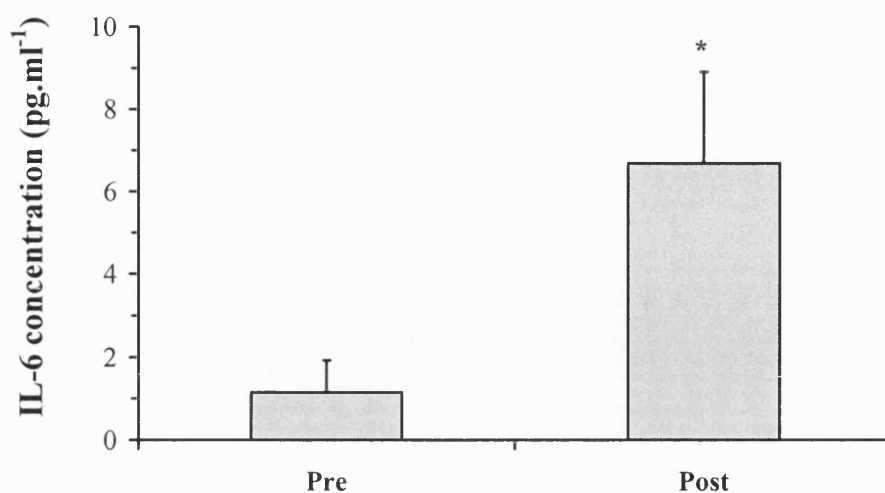


Figure 3.7 Plasma IL-6 concentration (pg.ml⁻¹) following a half marathon race. Pre: pre-exercise, Post: 1 h post-exercise. * denotes a significant difference in IL-6 concentration between 1 h post-exercise and pre-exercise ($P < 0.01$). Values are mean \pm S.D. (n=9).

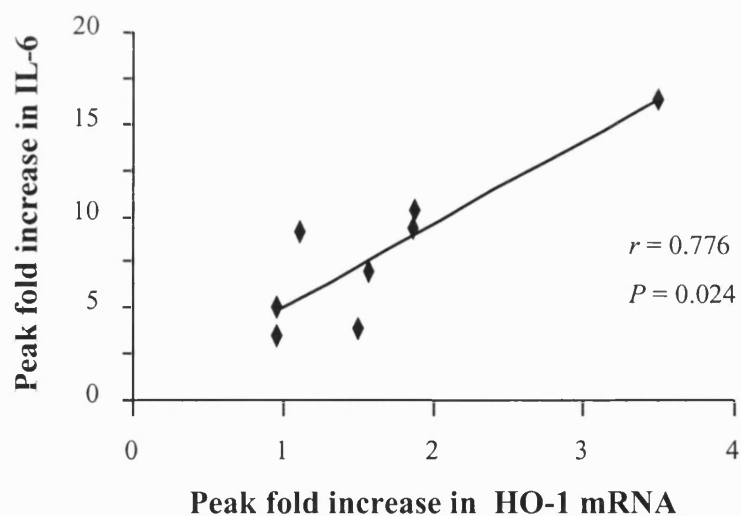


Figure 3.8 The relationship between the peak fold increase in plasma IL-6 concentration and peak HO-1 mRNA expression in MNCs following a half marathon race. (n=8).

3.4 Discussion

In the present investigation, exercise in the form of a competitive half marathon was used as the stimulus to induce HO-1 mRNA in human MNCs. The findings from this study reveal a large degree of inter-individual variability in the magnitude and time of peak HO-1 expression, which is consistent with previous reports assessing lymphocyte HO-1 mRNA expression following exercise (Thompson *et al.*, 2005). In the current investigation, an up-regulation of HO-1 mRNA at either 1 or 3 h following the race was observed in five out of the eight subjects. Although the subjects recruited for this investigation had been training specifically for this race for at least three months, their individual training history differed. This may account for reported differences in the basal regulation of HO-1 mRNA as previous studies reported endurance-trained individuals exhibited a lower HO-1 protein expression at rest (Niess *et al.*, 1999b). As training status could have an impact upon the resting level of HO-1, a comparison between the expression of HO-1 mRNA at rest with the peak fold change following exercise was made. From this it was possible to establish whether the magnitude of the response following exercise was affected by the expression of the gene at rest, and therefore contribute as a factor affecting inter-individual variability.

Thompson and colleagues (2005) reported an accumulation of lymphocyte HO-1 mRNA following a 75 min run at 70% $\dot{V}O_2\text{max}$, accompanied by an increase in HO-1 protein supporting the findings of Niess and co-workers (1999b). The differences between these two studies include the exercise protocol, the inclusion of a rest trial in one study, the determination of HO-1 mRNA and the use of western blotting compared to flow cytometry for HO-1 protein determination. Firstly, when using a competitive race as the exercise protocol the intensity and duration will be different for each subject. In contrast, a laboratory environment enables each subject to complete the exercise for the same duration and relative intensity. In the study conducted by Niess and colleagues (1999b), no information regarding the intensity of exercise was provided, therefore making comparisons with other exercise protocols difficult. In the present investigation, heart rate data was collected during the race in order to quantify the intensity at which subjects were exercising. In this study, the half marathon was completed in a mean time of 103 min at an intensity equivalent to 73% $\dot{V}O_2\text{max}$, indicating a prolonged and intense bout of exercise. The increase in HO-1 mRNA following exercise was not significant because a large inter-individual difference in the small sample population was found; although a moderate effect size was reported 1 h post-exercise.

In the present study, subjects had been training specifically for the competitive half marathon in the preceding months. It is possible that this could affect basal HO-1 either through an adaptation to training or through anticipation on the day of the event. This is an important finding as the elevated level at rest may have potentially masked any affect caused by the exercise itself since the basal level of certain genes is inversely related to their inducibility (Boshoff *et al.*, 2000). It is possible that a competitive event is likely to increase anxiety and circulating levels of hormones such as catecholamines and cortisol. Therefore, one of the major conclusions from the present study is that although the metabolic and immune responses to exercise in field or laboratory environments are generally the same, a competitive event may increase a number of stressors known to induce HO-1 prior to the event. Hence, competitive field-based races may be unsuitable when investigating gene up-regulation.

Intense physical exercise causes increases in the blood concentration of a number of stress hormones, including adrenaline, noradrenaline and cortisol (Hoffman-Goetz and Pedersen, 1994). In the current study, changes in the cell number of leukocytes were measured following exercise. Lymphocytes continuously recirculate throughout the body (Hay and Hobbs, 1977) and it is hypothesised that lymphocytes are recruited to the circulation from the spleen, lymph nodes and gastro-intestinal tract, with the extent of recruitment determined by the intensity of the stimulus (Pedersen and Hoffman-Goetz, 2000). In the present work, lymphocyte cell numbers fell below pre-exercise values during recovery, accompanied by large increases in neutrophil numbers with a smaller increase in monocytes. Changes to lymphocyte subsets were not examined and this should be taken into account in future work. It appears that catecholamines play an important role in the mediation of immuno-suppressive effects of exercise and could be the reason for apoptosis and the decline in cell number in lymphocytes. As the total lymphocyte concentration declines after exercise, the total *in vivo* lymphocyte function in the blood can be considered as 'suppressed' at this time (Pedersen and Hoffman-Goetz, 2000). Because of this decline, the modest but variable changes in the expression of HO-1 in lymphocytes may have an important role in the function of these cells.

An acute bout of exercise results in an inflammatory response, with the release of pro-inflammatory cytokines followed by regulatory anti-inflammatory cytokines (Pyne, 1994; Cannon and Blumberg, 2000; Moldoveanu *et al.*, 2001). The finding of markedly elevated levels of IL-6 after exercise in the current study is consistent with earlier investigations (Ostrowski *et al.*, 1998; Niess *et al.*, 1999b; Ostrowski *et al.*, 1999; Suzuki *et al.*, 1999; Moldoveanu *et al.*, 2000; Suzuki *et al.*, 2003; Thompson *et al.*, 2005). This inflammatory response is likely to compound oxidative stress initiated

during the exercise itself (Pyne, 1994). Both the previous studies that investigated HO-1 up-regulation following exercise reported changes in pro-inflammatory cytokines immediately post-exercise. A 4-fold change in IL-6 was observed by Thompson and co-workers (2005) with changes in IL-8 and TNF- α concentrations reported by Niess and colleagues (1999b). Furthermore, plasma levels of IL-8 were correlated with HO-1 expression in lymphocytes and neutrophils directly after exercise (Niess *et al.*, 1999b). In the present investigation, plasma IL-6 concentration was significantly greater than pre-exercise values and was positively correlated with peak HO-1 mRNA expression. However, the increase in plasma IL-6 concentration, from 1.2 to 6.7 pg.ml⁻¹ was relatively small compared to the change in IL-6 reported following other prolonged running events (Jeukendrup *et al.*, 2000; Nieman *et al.*, 2000; Suzuki *et al.*, 2003). Cytokines can induce HO-1 (Cantoni *et al.*, 1991) and it is possible that the induction of HO-1 could be the result of inflammatory-mediated production of reactive intermediates rather than a direct result of oxidative stress associated with mitochondrial respiration.

In conclusion, the main finding from this investigation is that HO-1 mRNA induction following intense exercise is highly variable between individuals. This variability was not explained by variation in the basal levels of HO-1 mRNA since there was no relationship between resting HO-1 and peak HO-1 induction. It is possible these responses reflect differences in antioxidant defences. Basal HO-1 mRNA expression seems to be elevated pre-competition and this could be either a specific response to preparatory training or a pre-competition elevation in HO-1. The induction of HO-1 seems to be related to an inflammatory response. The lack of response to exercise-induced oxidative stress is interesting and it is important to establish whether this is due to an adaptation to exercise or whether these are differences in the response to an oxidant challenge.

CHAPTER 4

REPRODUCIBILITY OF HO-1 PROTEIN INDUCTION IN HUMAN MONONUCLEAR CELLS FOLLOWING HYDROGEN PEROXIDE TREATMENT

4.1 Introduction

Haem oxygenase-1 is a marker of oxidative stress as it is up-regulated in response to a wide range of treatments causing oxidative stress. The exercise-induced expression of HO-1 is highly variable between individuals (Chapter 3) making it difficult to characterise the response to exercise and to use this as a model of oxidative stress. This may be a result of adaptations to regular exercise (Niess *et al.*, 1999b) or the presence of an HO-1 polymorphism (Yamada *et al.*, 2000). Adaptations to regular exercise enhance the antioxidant defence system and therefore the degree of oxidative stress encountered by individuals may vary and differ depending on the type of training that has been undertaken. However, it is not known whether lymphocytes from different individuals treated with an oxidant challenge *ex vivo* would respond in a similar manner.

The development of an *ex vivo* lymphocyte model may allow the HO-1 response to be better characterised, as a known concentration of an oxidant can be delivered directly to the cells of interest in contrast to a whole body stressor like exercise. It has been suggested that human lymphocytes provide an appropriate and convenient tool to use as an experimental model of oxidative stress (Rothfuss *et al.*, 1998; Rao *et al.*, 2003). Hydrogen peroxide (Khassaf *et al.*, 2003), hyperbaric oxygen treatment (HBO) (Rothfuss *et al.*, 1998), and heavy metals (Menzel *et al.*, 1998) have been used *ex vivo* to investigate oxidative stress in human lymphocytes. Haem oxygenase-1 is induced in lymphocytes in response to H₂O₂ treatment *in vitro* (Applegate *et al.*, 1991) and *ex vivo* (Marini *et al.*, 1996a; Rothfuss *et al.*, 2001). Furthermore, lymphocytes have been shown to be protected against H₂O₂-induced DNA damage 24 h after exposure to HBO treatment, indicating an adaptive response to prior oxidative stress (Speit *et al.*, 2000).

Marini and colleagues (1996a) observed that the level of protein expression of HO-1 in human lymphocytes following H₂O₂ treatment was not consistent between subjects. Before being able to employ this as a model of oxidative stress it is important to understand whether these differences are true inter-individual differences or related to day-to-day, random variation. To our knowledge, the intra-subject variation has not yet

been examined. Therefore, the aims of this study were to develop an *ex vivo* model of oxidative stress using H₂O₂ in order to (i) determine the time course of HO-1 protein expression in freshly isolated lymphocytes and monocytes, and (ii) determine the reproducibility of the HO-1 protein response to H₂O₂ treatment.

4.2 Methods

4.2.1 Subjects

Ten males participated in this study (Table 4.1). Physical activity was assessed by questionnaire (Appendix 2). Subjects were habitually active and completed 7 ± 2 h of physical activity per week (comprising both endurance and resistance exercise).

Table 4.1 Subject characteristics

Age (yrs)	Height (cm)	Mass (kg)	Σ Skinfolds (mm)
25.9 ± 3.9	177 ± 2	80.0 ± 3.2	32 ± 6

Values are mean \pm S.D. (n=10).

4.2.2 Experimental design

All subjects completed two trials, with each trial separated by two weeks (Figure 4.1). Subjects refrained from exercising and were not permitted to drink alcohol for 72 h prior to the trial day. During this time, subjects kept a record of their food and fluid intake (Chapter 2.4). This allowed subjects to repeat the diet recorded prior to the first trial in the 72 h prior to the second trial day, so that any changes were not a result of differences in diet. Daily energy intake was 2445 ± 494 kcal, with a macronutrient composition of $51 \pm 5\%$, $14 \pm 4\%$, and $34 \pm 6\%$ for carbohydrate, protein and fat, respectively.

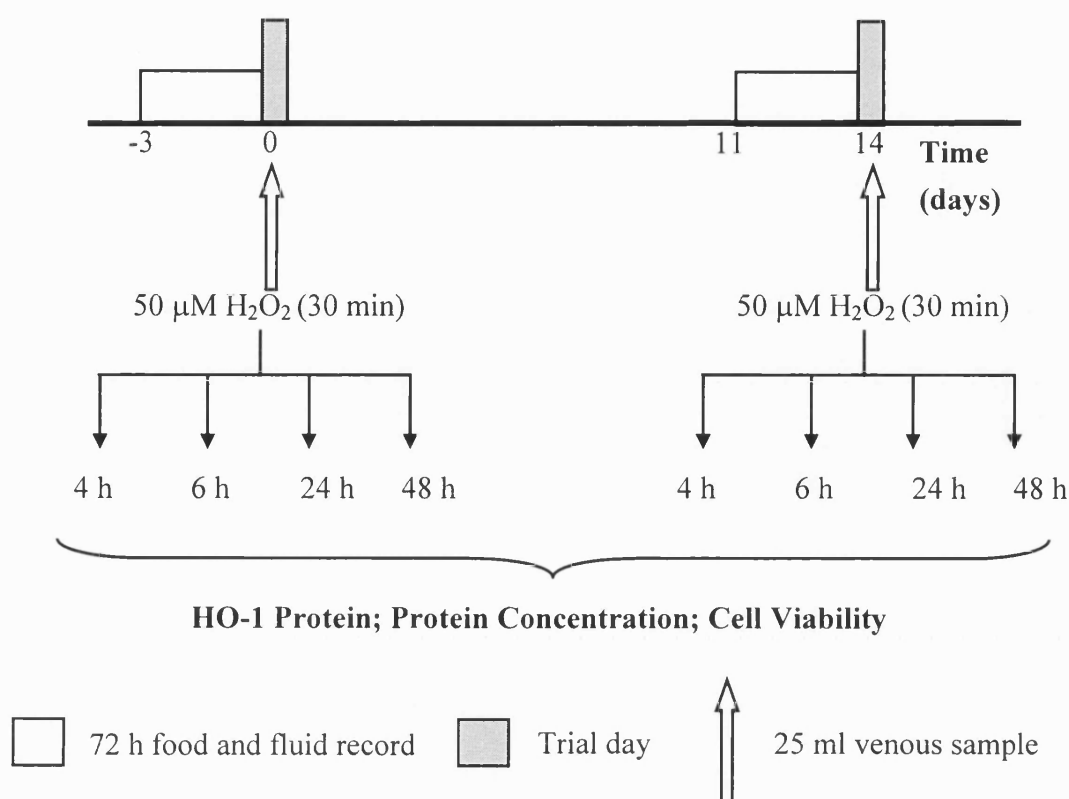


Figure 4.1 Experimental design of the *ex vivo* H_2O_2 treatment model. A venous sample was taken on two separate occasions, separated by two weeks. Mononuclear cells were harvested and treated *ex vivo* with H_2O_2 (30 min). Cells were left to recover for 4, 6, 24, and 48 h before determination of HO-1 protein.

4.2.3 Blood sampling and treatment

On the morning of the trial, subjects reported to the laboratory following an overnight fast. Subjects remained in a supine position for 15 min prior to blood sampling (Chapter 2.6). Twenty-five ml of whole blood was collected by venepuncture in the ante-cubital vein and placed in aliquots in EDTA coated tubes. Whole blood differential leukocyte counts, haemoglobin and haematocrit data were obtained using an automated haematology analyser (SF-3000, Sysmex UK Ltd., UK). Mononuclear cells were isolated and treated with H_2O_2 (Chapter 2.8.1) and recovered from incubation 4, 6, 24 and 48 h following treatment for analysis of HO-1 protein by flow cytometry (Chapter 2.9). Cell viability was assessed at these time points (Chapter 2.8.2) and protein concentration was determined using the Bradford assay (Chapter 2.6.4).

4.2.4 Statistical Analysis

The standard error of the measurement (SEM) is a practical index of measurement error (Bland and Altman, 1996b;1996a) and is proposed as the most useful estimate of reproducibility for individual-level applications (McHorney and Tarlov, 1995). For each data set, pair-wise plots of each subject's difference score (trial 1 minus trial 2) were plotted against the mean for the two trials (Bland and Altman, 1986). The plots were visually inspected for heteroscedasticity and the data analysed using the Shapiro-Wilks test of normality. These both showed that the data were not normally distributed and positively skewed. Therefore, a natural logarithm transformation was applied to all data points prior to analysis to normalise and stabilise the variance. The data were then back-transformed to provide information about the actual measurements (Bland and Altman, 1986).

The within-subject variation was derived using the SEM and the change in the mean. The SEM is the random error that arises from testing the same individual on two or more different occasions, and is calculated by dividing the standard deviation of the difference scores (SD_{diff}) by $\sqrt{2}$. The typical percentage error can be determined and is expressed as the percentage coefficient of variation (%CV) (Equation 4.1). This allows direct comparisons of reproducibility of measures irrespective of scaling (Hopkins, 2000a). The SEM expressed as a %CV was calculated using the formula:

$$\%CV = 100 (e^s - 1), \text{ where } s = SEM \quad \text{[Equation 4.1]}$$

The change in the mean is referred to as the bias and is estimated by the mean difference between trials. Systematic bias was assessed using a paired t statistic on each set of data. Where $P < 0.05$, there is evidence of systematic bias, implying that the difference between trial 1 and trial 2 may be consistently smaller or greater in the second trial.

In addition to calculating the SEM, re-test correlation was also determined (Pearson's product moment) for each pair of observations, indicating the reproducibility of the rank order of each subject's result. The fold change in HO-1 protein was expressed as the median fluorescence intensity (MFI) of the treated samples normalised to the MFI of the control samples. In order to examine whether differences between mean values existed between trials and over the time course, a two-way ANOVA was performed. A paired t statistic was performed in order to determine at which time point there were differences. Statistical significance was accepted at $P < 0.05$. Statistical calculations were performed using SPSS Version 11 (SPSS Inc., Chicago, USA) and a pre-formatted spreadsheet (Hopkins, 2000b). Values are expressed as mean \pm S.D.

4.3 Results

Time course of HO-1 protein induction

There was no difference in HO-1 protein induction between trial one and trial two ($P = 0.317$), although an effect of time was observed ($P = 0.010$). The time course of HO-1 protein induction showed that maximal HO-1 protein expression in response to H_2O_2 treatment occurred at 48 h following recovery (Figure 4.2). This was consistent in both trials with a mean fold change in lymphocyte HO-1 protein of 2.1 ± 0.9 ($P = 0.000$) and 1.9 ± 1.0 ($P = 0.008$) in trials 1 and 2, respectively. In trial 1, all ten subjects had maximum levels of HO-1 protein at 48 h with a mean fold change of 2.1 ± 0.9 ($P = 0.000$), whereas in trial 2, seven out of the ten subjects peaked at 48 h with a peak mean fold change of 2.0 ± 0.9 ($P = 0.000$). Of the three subjects whose response differed in trial 2, maximal fold change in HO-1 was at 24 h in two individuals and at 6 h in the other.

In monocytes, the effect of time and the response to H_2O_2 was similar to lymphocytes but less pronounced ($P = 0.051$) (Figure 4.2). At 48 h following treatment, the fold change in HO-1 protein was 1.3 ± 0.4 in trial 1 ($P = 0.022$) and 1.5 ± 0.6 in trial 2 ($P = 0.018$). Six individuals had maximum levels of HO-1 in monocytes at 48 h in trial 1 compared to five in trial 2. In trial 1, the number of individuals with maximum HO-1 expression at 24 h was two, increasing to five in trial 2, whereas two individuals had maximum monocyte HO-1 induction at 6 h in trial 1 alone. The maximum fold change in monocytes, irrespective of time, was 1.4 ± 0.3 ($P = 0.002$) and 1.6 ± 0.5 ($P = 0.000$) in trials 1 and 2, respectively.

The magnitude of the maximal HO-1 response in lymphocytes differed between subjects ranging from a 1.4-fold change to a 4.6-fold change from control values. An example of this range is depicted in Figure 4.3. Inter-individual differences were less pronounced in monocytes, ranging from a 1.2-fold change to a 2.6-fold change in maximal HO-1 protein compared to control values.

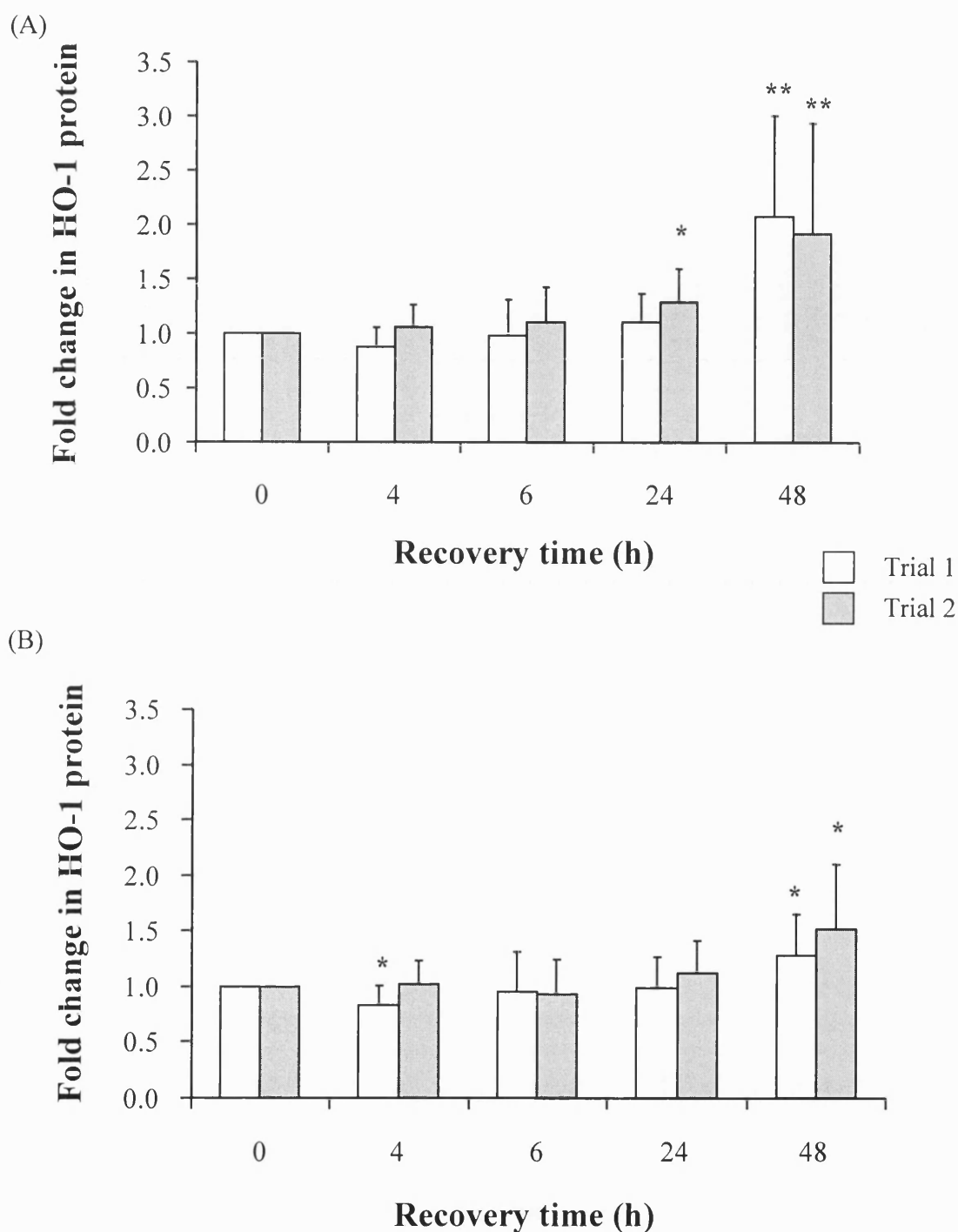
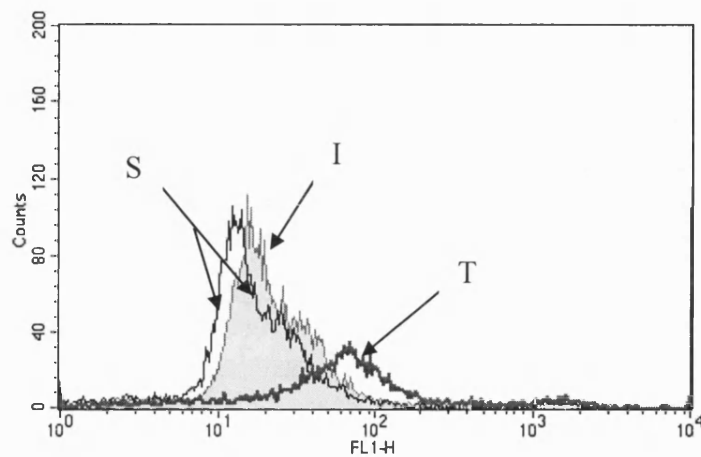


Figure 4.2 The time course of HO-1 protein induction following H_2O_2 treatment in lymphocytes (A) and monocytes (B). The fold change is the MFI of the treated samples normalised to the MFI of the sham-treated controls. 0 h: untreated control. ** denotes a significant difference between time and untreated control ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=10).

(A)



(B)

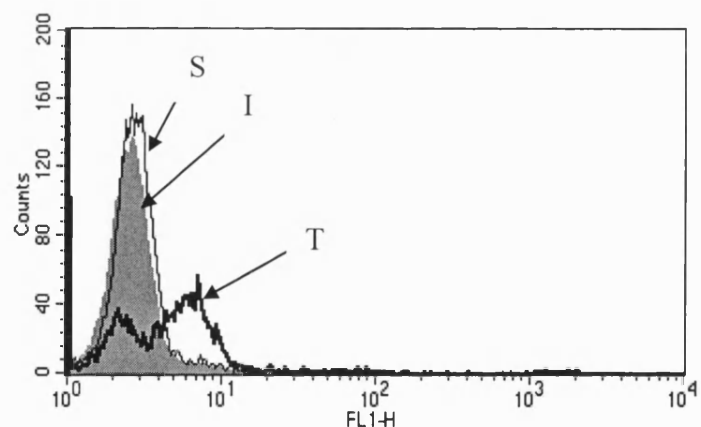


Figure 4.3 A representative flow cytometric analysis of HO-1 protein induction in human lymphocytes at 48 h in two different subjects illustrating the varied response to H_2O_2 treatment. A 4.6-fold change is shown in (A) with a 1.6-fold change observed in (B). I represents the isotype control, S represents the sham-treated sample and T represents H_2O_2 treatment.

Reproducibility analyses

Total leukocyte population number was analysed in both trials with a small systematic bias in both lymphocyte and monocyte populations. The SEM as a %CV was 5% and 11% in the lymphocyte and monocyte populations, respectively (Table 4.2).

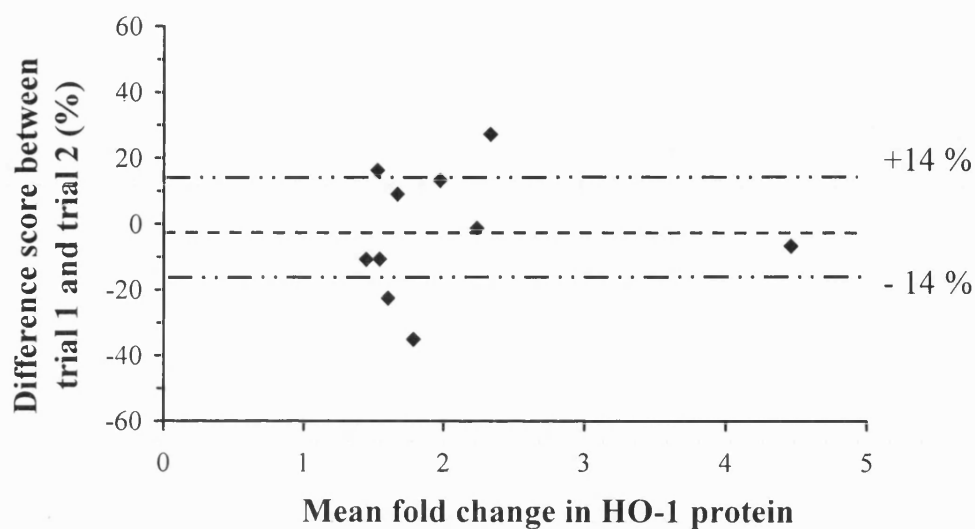
Table 4.2 Reproducibility data for total leukocyte and differential population cell counts ($\times 10^9 \cdot l^{-1}$) taken from venous blood for trials 1 and 2

Population ($\times 10^9 \cdot l^{-1}$)	Trial 1	Trial 2	% change in mean (95% CI)	SEM (%CV) (95% CI)
WBC	4.5 ± 1.0	4.5 ± 1.0	-2 (-7 to 3)	6 (4 to 11)
Neutrophils	2.3 ± 0.8	2.2 ± 0.8	-4 (-13 to 7)	11 (8 to 22)
Lymphocytes	1.8 ± 0.3	1.7 ± 0.2	-3 (-7 to 2)	5 (3 to 9)
Monocytes	0.4 ± 0.1	0.4 ± 0.1	-5 (-14 to 6)	11 (8 to 23)

WBC: total white blood cells. Values are mean \pm S.D. (n=10).

The reproducibility of the HO-1 protein response between trials was assessed at all time points. There was no significant difference between the mean HO-1 protein for any of the pairs of trials, in either lymphocytes or monocytes, showing negligible systematic bias between trials 1 and 2 (Figure 4.4). Systematic bias was largest following 4 h recovery and tended to be smaller in lymphocytes than in monocytes (Tables 4.3 and 4.4). The SEM (%CV) represents the variability of measurements for individual subjects in a population. Table 4.3 shows that the SEM varied across the recovery time following H_2O_2 treatment. In general, the SEM was similar in both lymphocytes and monocytes at each recovery time. The SEM was largest following 6 h recovery at 44% and 49% in lymphocytes and monocytes, respectively. However, the SEM decreased to 30% and 29% at 48 h in lymphocytes and monocytes, respectively. The maximum fold change in HO-1, irrespective of time, showed good reproducibility with a SEM of 14% in lymphocytes and 18% in monocytes.

- (A) - - - - - Systematic bias (change in the mean)
 - · - · - · - SEM represented as %CV (with respect to the change in the mean)



(B)

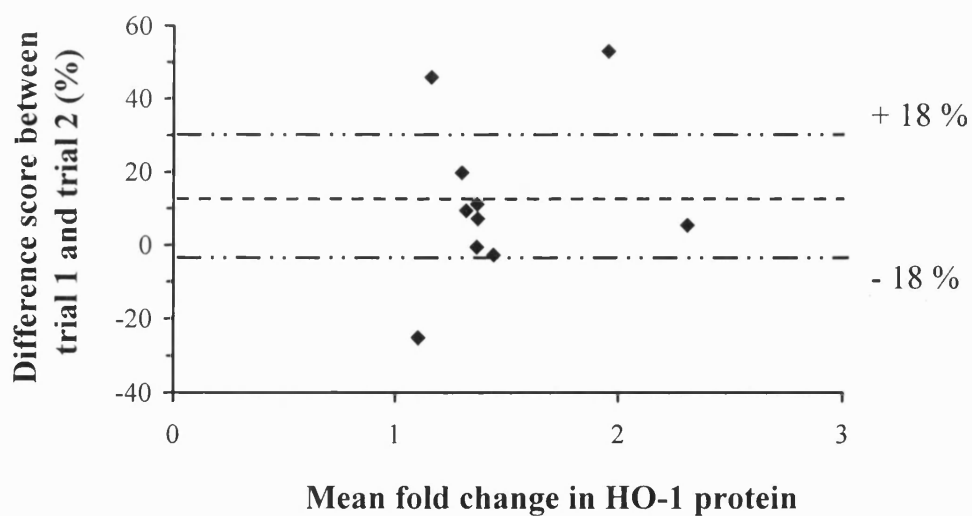


Figure 4.4 Reproducibility plots of peak HO-1 protein induction in lymphocytes (A) and monocytes (B). The data represents the difference score in HO-1 protein between trials 1 and 2 against the mean fold change in HO-1 protein for each subject. (n=10).

Table 4.3 Reproducibility data for lymphocyte HO-1 protein expression at basal levels, peak induction and 4, 6, 24 and 48 h following H₂O₂ treatment

Parameter	Trial 1	Trial 2	% change in mean (95 % CI)	SEM (% CV) (95% CI)	Correlation coefficient (<i>r</i>)
Lymphocytes					
Peak	2.1 ± 0.9	2.0 ± 0.9	-2 (-14 to 12)	14 (10 to 30)	0.92**
48 h	2.1 ± 0.9	1.9 ± 1.0	-12 (-32 to 15)	30 (23 to 73)	0.92**
24 h	1.1 ± 0.3	1.3 ± 0.3	17 (-1 to 42)	20 (15 to 43)	0.84**
6 h	1.0 ± 0.3	1.1 ± 0.3	16 (-19 to 70)	44 (36 to 124)	-0.28
4 h	0.9 ± 0.2	1.1 ± 0.2	22 (-1 to 55)	25 (19 to 57)	0.37
Basal	1.0 ± 0.2	0.9 ± 0.1	-9 (-21 to 4)	11 (7 to 27)	0.57

** denotes a significant correlation coefficient ($P < 0.01$). Values are mean ± S.D. (n=10 for all time points other than basal, where n=6).

Table 4.4 Reproducibility data for monocyte HO-1 protein expression at basal levels, peak induction and 4, 6, 24 and 48 h following H₂O₂ treatment

Parameter	Trial 1	Trial 2	% change in mean (95 % CI)	SEM (% CV) (95% CI)	Correlation coefficient (<i>r</i>)
Monocytes					
Peak	1.4 ± 0.3	1.6 ± 0.5	13 (-3 to 34)	18 (13 to 38)	0.66*
48 h	1.3 ± 0.4	1.5 ± 0.6	12 (-14 to 49)	29 (21 to 73)	0.56
24 h	1.0 ± 0.3	1.1 ± 0.3	15 (-8 to 46)	25 (19 to 59)	0.46
6 h	0.9 ± 0.4	0.9 ± 0.3	1 (-32 to 52)	49 (40 to 146)	0.05
4 h	0.8 ± 0.2	1.0 ± 0.2	23 (0 to 59)	26 (19 to 60)	0.24
Basal	1.1 ± 0.2	1.1 ± 0.1	-6 (-17 to 6)	10 (7 to 24)	0.59

* denotes a significant correlation coefficient ($P < 0.05$). Values are mean ± S.D. (n=10 for all time points other than basal, where n=6).

Re-test correlation for peak HO-1 induction was statistically significant in lymphocytes ($r = 0.92$, $P = 0.000$) and in monocytes ($r = 0.66$, $P = 0.036$) (Tables 4.3 and 4.4). This was similar at 48 h in lymphocytes ($r = 0.84$, $P = 0.002$) and smaller in monocytes ($r = 0.46$, $P = 0.214$).

The basal level of HO-1 protein was also determined although due to technical difficulties, the basal level of HO-1 protein in both trials was only available for six subjects. In lymphocytes and monocytes ($n=6$), the %CV was $-9\% \pm 11\%$ and $-6\% \pm 10\%$, respectively (Tables 4.3 and 4.4). This indicates that the basal levels of HO-1 protein are reproducible when individuals refrain from physical activity and follow a replicated diet for 72 h prior to measurement.

Cell Viability

Cell viability following H_2O_2 treatment was measured at all time points (Figure 4.5). As expected, H_2O_2 treatment resulted in reduced cell viability in comparison to the respective sham-treated control and viability decreased with increasing recovery time.

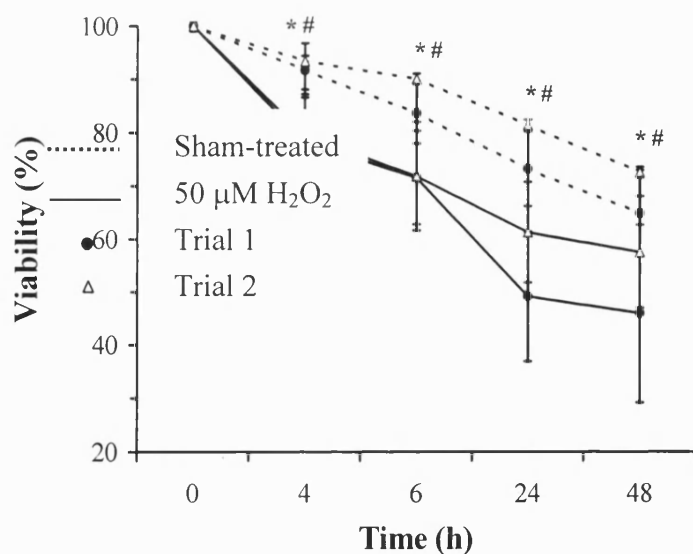


Figure 4.5 Cell viability of MNCs before treatment and 4, 6, 24 and 48 h following H_2O_2 . 0 h: untreated control. Values are mean \pm S.D. ($n=10$). * In trials 1 and 2, values following H_2O_2 are different to 0 h ($P < 0.01$), # In trials 1 and 2, values for viability following H_2O_2 are different to sham-treated control in respective trial ($P < 0.01$).

The change in the mean and the SEM, as a %CV, increased with time and was larger in the treated samples than the sham-treated controls (Table 4.5).

Table 4.5 Reproducibility data for mononuclear cell viability at 4, 6, 24 and 48 h following H₂O₂ treatment

Parameter	Trial 1 (%)	Trial 2 (%)	% change in mean (95 % CI)	SEM (% CV) (95% CI)	Correlation coefficient (<i>r</i>)
4 h sham	92 ± 5	93 ± 5	2 (-4 to 8)	6 (4 to 12)	-0.05
4 h treated	78 ± 10	78 ± 7	2 (-9 to 14)	12 (9 to 25)	0.00
6 h sham	84 ± 10	90 ± 6	8 (-1 to 19)	10 (7 to 19)	0.28
6 h treated	72 ± 9	72 ± 10	0.2 (-15 to 17)	17 (12 to 36)	-0.38
24 h sham	73 ± 1	81 ± 7	12 (-3 to 31)	16 (12 to 34)	-0.18
24 h treated	49 ± 12	61 ± 10	28 (5 to 67)	26 (19 to 59)	0.21
48 h sham	65 ± 16	73 ± 8	15 (-3 to 38)	19 (14 to 42)	0.29
48 h treated	46 ± 17	58 ± 11	30 (6 to 73)	27 (21 to 65)	0.24

Values are mean ± S.D. (n=10).

4.4 Discussion

The present study supports previous research showing an *ex vivo* up-regulation of HO-1 protein in lymphocytes following H₂O₂ treatment (Marini *et al.*, 1996a). This is the first work investigating the within-subject variation of the HO-1 protein response to H₂O₂. In addition, it confirms reports of large inter-individual variations in the ability to increase lymphocyte HO-1 protein in response to oxidants (Marini *et al.*, 1996a; Menzel *et al.*, 1998). The main finding is that maximal expression of HO-1 protein is a reproducible measure for reporting the response of this enzyme to H₂O₂ treatment.

The physiological concentration of H₂O₂ in human plasma has been reported to be 4-5 μ M (Yamamoto *et al.*, 1987) and this increases under inflammatory conditions. Some studies have found levels of H₂O₂ in human urine as high as 100 μ M (Long *et al.*, 1999a), with many common beverages containing H₂O₂ at concentrations greater than 100 μ M (Long *et al.*, 1999b). In the present investigation, MNCs were treated with a concentration of 50 μ M H₂O₂. The concentration of H₂O₂ used was sufficient to up-regulate HO-1 protein in a time-dependent manner with increased levels of the protein from 24 h. Maximum levels of HO-1 protein expression were observed 48 h following H₂O₂ treatment in lymphocytes and were equivalent to a 2-fold increase.

Previous studies have demonstrated an increase in HO-1 protein in lymphocytes as early as 6 h, with HO-1 expression maintained at least 18 h following H₂O₂ treatment (Marini *et al.*, 1996a). Other investigators have shown that in human lymphocytes using a non-genotoxic HBO treatment as the model of oxidative stress, induction of HO-1 protein increases in a time-dependent manner and was maximum at 24 h (Speit *et al.*, 2000; Rothfuss and Speit, 2002). Furthermore, these authors showed that increased HO-1 induction protected lymphocytes from oxidative DNA damage induced by a repeated HBO exposure or by treatment with H₂O₂, supporting the role of HO-1 as cytoprotective. However, in contrast to these findings, some authors have reported that HO-1 protein was not detectable *in vivo* in lymphocytes at basal level or following different types of oxidative stress (Yachie *et al.*, 2003). Although in the present study, monocytes showed a similar response to treatment as lymphocytes, with the induction of HO-1 protein greatest 48 h following the stimulus. There is little evidence in the literature investigating H₂O₂ treatment and HO-1 protein induction in monocytes, however, treatment of monocytes with alternative oxidants has shown increased levels of HO-1 protein (Pinot *et al.*, 1997; Bornman *et al.*, 1999). The present findings demonstrate a delayed induction of HO-1 protein in comparison to other studies

investigating HO-1 protein induction in lymphocytes (Marini *et al.*, 1996a). However, this time point for maximal HO-1 protein induction has been reported in osteoarthritic chondrocytes (Fernandez *et al.*, 2003) and mouse peritoneal macrophages (Vicente *et al.*, 2003a). These authors suggest that the delayed induction of HO-1 may reflect an autocrine response to oxidative stress (Fernandez *et al.*, 2003; Vicente *et al.*, 2003a), although there is little direct evidence to support this contention at the present time.

Several studies have shown a large inter-individual variation in the induction of HO-1 protein in humans (Marini *et al.*, 1996a; Menzel *et al.*, 1998). It is possible that the variation in the induction of HO-1 is a result of the presence of a polymorphism in the gene associated with susceptibility to oxidative-stress mediated diseases (Hirai *et al.*, 2003). For example, the microsatellite polymorphism GT (n) repeat is present in the human HO-1 gene promoter and is associated with increased risk of emphysema (Yamada *et al.*, 2000). Undoubtedly, the presence of a polymorphism in the human HO-1 gene could cause inter-individual variation of HO-1 activity, with the absence of the GT (n) repeat in rat and mouse HO-1 genes accounting for the interspecies variation in the regulation of HO-1 expression (Shibahara *et al.*, 2002). Alternatively, the variation could be attributed to a number of factors such as health status, exercise, general stress and diet, although they are more likely to have implications for the within-subject variation and these factors were tightly controlled in the current investigation. Ultimately, from an experimental perspective, it is the ability to reproduce the response on different days that is critical.

Re-test correlation reflects the reproducibility of the rank order of the subjects on re-testing. In lymphocytes and monocytes this was statistically significant ($r = 0.92$, $P = 0.000$ and $r = 0.67$, $P = 0.036$ in lymphocytes and monocytes, respectively). Therefore, those subjects who responded highest in trial 1 remained high in trial 2 and were placed in a similar positioning in terms of order. Although re-test correlation is a good measure of reproducibility, it comprises both between-subject and within-subject variation (Hopkins *et al.*, 1999) and is only applicable to individuals similar to those sampled. The within-subject error is a better measure of reproducibility as it can be estimated from a sample of individuals not representative of a population (Hopkins, 2000a).

In the present study, the within-subject variation of the HO-1 response to H₂O₂ treatment decreased in a time-dependent manner. There was no significant difference between the two trials at any time point, thus suggesting negligible systematic bias. The number of responders to the treatment at 4 and 6 h was very small and not consistent

among the sample population increasing both the systematic bias and the SEM. The SEM is usually larger than the bias and represents the random error due to biological or mechanical factors inherent in the measurement tool (Hopkins, 2000a). An ideal measure would have both a small systematic bias and a small SEM. The number of individuals mounting a response to the treatment through increased HO-1 up-regulation increased with time of recovery, therefore minimising the within-subject variation in the lymphocyte HO-1 response to treatment. All ten subjects had a maximum induction of HO-1 protein at 48 h in trial 1 and seven of these in trial 2. At 48 h, the change in the mean \pm SEM, as a %CV, was $-12\% \pm 30\%$ and $12\% \pm 29\%$ in lymphocytes and monocytes, respectively. Additionally, as the majority of subjects experienced maximal HO-1 protein induction at this time in both cell populations, this would be the most appropriate time point to use in further investigations. The change in the mean \pm SEM can be expressed in raw values as a fold change. At 48 h, this was -0.16 ± 0.39 fold change and 0.21 ± 0.37 fold change in lymphocytes and monocytes, respectively. Therefore, if this time point is used in future investigations where an intervention is employed, the researcher can estimate that any change in HO-1 protein expression that is greater than 30% or 0.40 fold change, with regards to the change in the mean between the two trials, is related to the treatment and not to random error.

The peak response had the smallest change in the mean and SEM, as a %CV, ($-2\% \pm 14\%$ and $13\% \pm 18\%$ in lymphocytes and monocytes, respectively). There was a negligible change in the mean and relatively small random error in lymphocytes which was -0.03 ± 0.26 when expressed as a fold change. In monocytes, the change in the mean was larger and the SEM was the same as in lymphocytes (0.20 ± 0.26 fold change). Altered gene expression is a sensitive marker of oxidative stress *in vitro* (Tyrrell and Basu-Modak, 1994; Griffiths *et al.*, 2002) and mononuclear cell HO-1 protein expression fulfils the criteria as a valid biomarker of oxidative stress since the measured product is specific, sensitive and reproducible (Offord *et al.*, 2000; Griffiths *et al.*, 2002). Accordingly, the results presented here confirm that the induction of HO-1 protein is a reproducible measure of the response to oxidative stress in lymphocytes.

In conclusion, the present investigation has provided the first evidence detailing the within-subject variation of HO-1 protein induction in response to H₂O₂ treatment. This study shows that the reproducibility of the response changes over time and is most reproducible when there is maximum induction of the gene. This method is, therefore, a suitable model to use following exposure to (i) a prior stimulus known to induce HO-1, or (ii) a modifying factor which may alter the basal level of HO-1 and, hence, the pattern of expression of the enzyme upon further treatment. In the next chapter, this model is

applied before and after demanding exercise in order to establish whether prior exercise affects the ability of lymphocytes to respond to a subsequent oxidant challenge.

CHAPTER 5

THE EFFECT OF EXERCISE ON EXPRESSION OF HO-1 IN HUMAN MONONUCLEAR CELLS IN RESPONSE TO SUBSEQUENT OXIDATIVE STRESS

5.1 Introduction

Lymphocytes are immune cells that are continually exposed to ROS, and therefore, the manner in which they react to ROS is important in their ability to function. Strenuous long duration exercise causes oxidative damage to tissues resulting in an increase in plasma lipid hydroperoxides (Alessio *et al.*, 2000; Davison *et al.*, 2002; Nieman *et al.*, 2003), lymphocyte DNA damage (Tsai *et al.*, 2001; Mastaloudis *et al.*, 2004b), and increased plasma protein carbonyls following exercise (Radak *et al.*, 1997; Lee *et al.*, 2002).

Acute exercise induces a transient inflammatory response and this may serve as an important secondary source of free radical production during the recovery period following heavy exercise (Ji, 1999). This response is characterised by the release of pro-inflammatory cytokines with plasma IL-6 concentrations peaking immediately post-exercise (Pedersen and Toft, 2000; Nieman *et al.*, 2001; 2003). High-intensity exercise induces apoptosis in lymphocytes (Mars *et al.*, 1998) and there are conflicting findings regarding whether apoptosis is implicated in the decline in lymphocyte concentration in the hours following exercise (Mars *et al.*, 1998; Steensberg *et al.*, 2002). Furthermore, lymphocyte proliferation is depressed 2 h post-exercise (MacNeil *et al.*, 1991; Nielsen and Pedersen, 1997; Mitchell *et al.*, 2002). Consequently, the damage caused by strenuous exercise may have important implications for lymphocyte function in the hours following exercise, particularly for individuals who engage in successive exercise sessions.

Therefore, demanding exercise may have a negative impact on lymphocytes as it causes an excessive increase in the production of ROS. The oxidative damage induced following exercise may increase the susceptibility to subsequent oxidative stress and this has led to suggestions that individuals participating in these activities may benefit from antioxidant supplementation (Sen, 1995; Ashton *et al.*, 1998). This scenario could provide an ideal model to manipulate the response to oxidative stress using antioxidants. Superimposing the *ex vivo* model (Chapter 4) upon exercise-induced oxidative stress

provides a method for examining the response of lymphocytes to subsequent oxidative stress, replicating the process that may occur following acute exercise *in vivo*. Hence, the aim of the present investigation was to determine whether (i) HO-1 is induced by demanding exercise and (ii) exercise increases the susceptibility of lymphocytes to subsequent H₂O₂ exposure and changes the HO-1 response to this secondary oxidant challenge.

5.2 Methods

5.2.1 Subjects

Eight male students participated in this study (Table 5.1). Physical activity was assessed by questionnaire (Appendix 2). The exercise was intermittent in nature and all subjects recruited were familiar with intermittent activities such as football, squash and rugby, completing on average 3 ± 1 h of activity per week (mean \pm S.D.) Subjects were excluded from the investigation if they smoked, took regular prescribed medication or food supplements or were endurance-trained.

Table 5.1 Subject characteristics

Age (yrs)	Height (cm)	Mass (kg)	$\dot{V}O_2\text{max}$ (ml.kg ⁻¹ .min ⁻¹)	HR _{max} (beats.min ⁻¹)	Σ Skinfolds (mm)
24.5 ± 3.8	180 ± 6	78.8 ± 8.9	58 ± 6	200 ± 9	41 ± 13

Values are mean \pm S.D. (n=8).

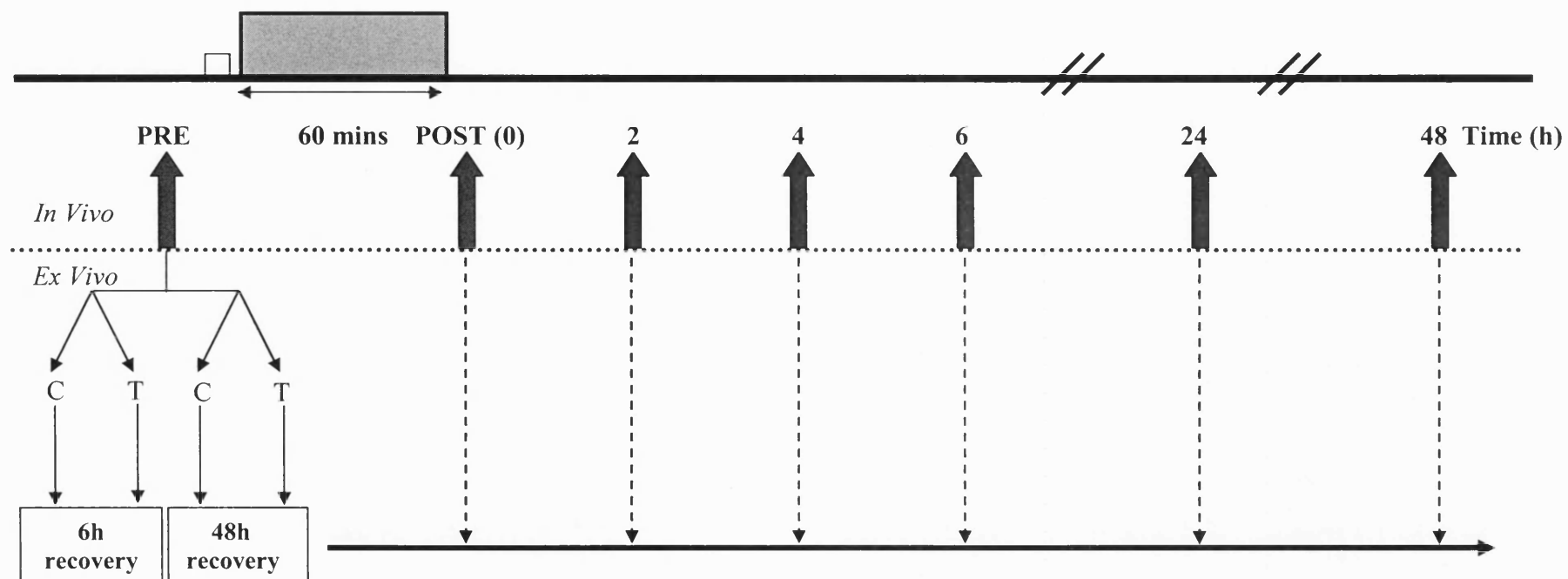
5.2.2 Experimental design

Each subject completed two trials, one exercise (Ex) and one control (Rest), separated by two weeks with the order of the trials randomised. Subjects were asked to record their food and fluid intake during the 72 h prior to the main trial, which was replicated prior to the second trial (Chapter 2.4). Prior to the main trials, all individuals completed preliminary testing to establish their $\dot{V}O_2\text{max}$ (Chapter 2.3) and to determine their individual running speeds for the intermittent exercise protocol (IEP). The IEP consisted of a 60 min run divided into four 15 min blocks comprised of bouts of speeds equivalent to 65, 85 and 100% $\dot{V}O_2\text{max}$ (Figure 5.1). This protocol was developed so that a high-intensity exercise could be used as a model to induce changes in HO-1 mRNA that are greater than levels previously demonstrated with other protocols. As intermittent

h samples (1771 kcal, 62% carbohydrate, 12% protein and 26% fat). This diet was replicated in the control trial, during which subjects sat in the lab resting for 60 min, with gas samples and capillary blood being collected at the same times as in the exercise trial.

5.2.3 Blood sampling and treatment

On the day of a main trial, subjects reported to the laboratory following an overnight fast. A resting venous sample was taken from the ante-cubital vein following rest in a supine position for 15 min (Chapter 2.6). Approximately 25 ml of blood was collected and placed in aliquots in EDTA coated tubes (Chapter 2.6). Plasma was removed and stored at -70 °C prior to analysis of plasma interleukin-6 (IL-6) and interleukin-10 (IL-10) concentrations (Chapter 2.11). Mononuclear cells were harvested and incubated for 45 min to separate monocytes and lymphocytes before lysis in TRIZOL (Chapter 2.10). HO-1 mRNA was determined using real-time RT-PCR (Chapter 2.10.2). Approximately 12×10^6 cells were resuspended in PBS for treatment with H_2O_2 and left to recover for 6 and 48 h (Chapter 2.8.1). Following recovery from H_2O_2 cells were pelleted and fixed in 70% ice cold ethanol and left at 4 °C for three days prior to analysis of HO-1 by flow cytometry (Chapter 2.9). Approximately 12×10^6 cells were set aside for immunophenotyping. Cell viability was assessed after the MNC separation procedure (Chapter 2.8.2) along with the determination of protein concentration (Chapter 2.6.4). The *ex vivo* treatment was performed prior to exercise and rest and 0, 2, 4, 6, 24, and 48 h post-exercise and post-rest (Figure 5.2).



KEY:



25 ml venous sample



5 min warm - up



IEP/rest (60 min)

C Control (sham-treated)

T 50μM H₂O₂ treatment (30 min)



Repeat *ex vivo* H₂O₂ treatment

Figure 5.2 Experimental design investigating the effect of exercise on expression of HO-1 in human MNCs in response to subsequent exposure to H₂O₂.

5.2.4 Immunophenotyping

The direct immunofluorescence method of staining was used to determine lymphocyte subsets from a MNC population using a dual combination of monoclonal antibodies conjugated to FITC or R-phycoerythrin (PE). Samples were analyzed using 488 nm excitation and detection in the green fluorescence channel for FITC (FL1: 530 ± 30 nm bandpass filter) and orange fluorescence channel for PE (FL2: 585 ± 42 nm bandpass filter). Electronic compensation was set to correct for spectral overlap in both FITC and PE emissions. When the cells are correctly compensated the population medians of labelled cells should align parallel to both axes (Figure 5.3).

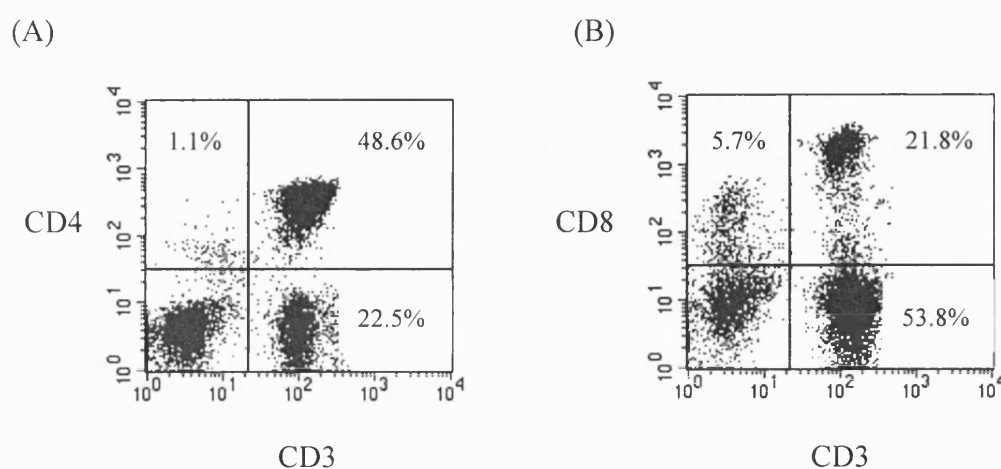


Figure 5.3 Dual parameter dot plot of FL2 *versus* FL1, determining the percentage of CD4 (A) and CD8 (B) T cells in a MNC population. T cells are positive for CD3/CD4 and are located in the upper right quadrant of (A), with CD3/CD8 cells located in the upper right quadrant of (B).

Briefly, 1×10^6 cells were incubated with 20 μ l of conjugate (CD3-FITC/CD4-PE; CD3-FITC/CD8-PE) in the dark for 30 min at room temperature. After incubation, 2 ml diluent was added and the cells centrifuged (170 g, 5 min at room temperature). The supernatant was aspirated and the cell pellet suspended in a fixative solution of 2% paraformaldehyde in PBS. An isotype control was performed with FITC mouse IgG1 and PE mouse IgG2a monoclonal antibodies to determine background fluorescence. Cells were kept on ice until analysis by flow cytometry. Ten thousand events of the lymphocyte population were collected using a Becton Dickinson FACScan (Cellquest version 3.3 software, Belgium). Gates based on cell size and granularity were used to distinguish between lymphocyte and monocyte populations for fluorescence analysis.

The data was analysed using two-dimensional dot plots of FL2 *versus* FL1 fluorescent profiles.

5.2.5 Statistical Analysis

Haem oxygenase-1 mRNA and protein in response to exercise (Ex) or rest were normalised to the pre-trial time point. In the *ex vivo* model, HO-1 protein in the treated sample was normalised to the respective sham-treated control, and then expressed relative to the pre-trial time point. For each trial, the change in HO-1 mRNA and protein expression was determined at each time point following exercise/rest in relation to the pre-exercise/rest sample to produce a change score for each subject [ChangeEx0 – ChangeRest0 = (0 h post-Ex minus pre-Ex) minus (0 h post-rest minus pre-rest), where 0 represents the 0 h post-trial time point]. Paired *t* statistics of the change scores for each parameter were performed.

The data were not normally distributed, therefore, a natural log transformation was applied. This was followed by back-transformation of the log effects into factors. The difference in the mean of the exercise and rest trials change scores was calculated using effect size (ES) (Chapter 3.2.3), using the control trial SD as the denominator. This provided additional information regarding the practical significance as the ES represents the magnitude of the difference between the change score (ChangeEx minus ChangeRest) over the time course. Values lying between 0.2 to 0.49, and 0.5 to 0.79 were considered small and moderate effects, whereas ES greater than 0.8 indicated large differences (Cohen, 1988).

The clinical significance of the change in HO-1 expression was assessed from *Q* value calculations. This statistic represents the probability that the true effect of HO-1 induction with exercise was at least as great as the minimum worthwhile effect (Froehlich, 1999). This was derived from testing a hypothesis of an arbitrary minimum effect rather than a hypothesis of no effect as in a *t* test (Equation 5.1) (Froehlich, 1999). In this study, the minimum worthwhile effect (δ) (smallest worthwhile difference) was regarded at an ES of 0.2, in order to estimate the % chance that the true value was at least a ‘small’ effect. In this Chapter, the term ‘clinical’ significance will be referred to as ‘biological’ significance because the ‘clinical’ utility of this measurement is not known.

$$t_Q = [\delta - (\bar{M}_1 - \bar{M}_2)] / SE \quad [\text{Equation 5.1}]$$

Where, Q = probability that $T > t_Q$ SE = standard error

Traditionally, when clinical significance is employed, the % chance that the true value is at least as great as the minimum worthwhile effect is defined as a positive, trivial or negative change. In this Chapter, a positive or negative change will be defined as an increase or decrease, respectively. This can be interpreted in both a quantitative and qualitative form (very unlikely: < 5%, unlikely: < 25%, possible: < 75%, likely: < 95%, very certain: > 95%). For a change to be practically significant, the estimate that the % chance was greater than or equal to 75% was accepted, indicating a 'likely' or 'probable' chance that the observed effect was true.

Pearson's product moment correlations were calculated comparing the peak fold increase in IL-6 and IL-10 concentration with the peak fold increase in HO-1 mRNA in the exercise trial. The statistical calculations described were performed using a pre-formatted spreadsheet (Hopkins, 2003) in addition to SPSS version 11 (SPSS Inc., Chicago, USA). Significance was accepted at the 5% level. Values are presented as mean \pm S.D. The data represent values for eight subjects, except in the *ex vivo* model at 48 h where values represent seven subjects due to problems with the pre-trial *ex vivo* treatment in one subject.

5.3 Results

Exercise Protocol

The overall intensity of the IEP was predicted to be 75% $\dot{V}O_{2\text{max}}$. During exercise, expired air samples could only be collected once per block in the 65% $\dot{V}O_{2\text{max}}$ stage to ensure the actual intensity of the protocol was equivalent to 65%. This was $67 \pm 2\%$ in block one, increasing to $69 \pm 4\%$ in block four. During these lower intensity phases, the exercise resulted in an 11-fold increase in oxygen consumption when compared to all resting values (Table 5.2). As expected, the exercise produced significantly different changes in blood lactate and blood glucose concentrations, oxygen consumption and heart rate when compared to the resting trial ($P < 0.05$) (Table 5.2).

Table 5.2 A comparison of the changes in blood lactate and blood glucose concentrations, heart rate and oxygen consumption at rest vs. exercise

Rest Trial					Exercise Trial			
Block Number	Blood Lactate (mmol.l ⁻¹)	Blood Glucose (mmol.l ⁻¹)	Heart Rate (beats.min ⁻¹)	$\dot{V}O_2$ (ml.kg ⁻¹ .min ⁻¹)	Blood Lactate (mmol.l ⁻¹)	Blood Glucose (mmol.l ⁻¹)	Heart Rate (beats.min ⁻¹)	$\dot{V}O_2$ (ml.kg ⁻¹ .min ⁻¹)
Rest				4.0 ± 0.8				3.8 ± 0.8
1	0.8 ± 0.3	4.4 ± 0.3	61 ± 4	3.6 ± 1.5	4.5 ± 1.3**	4.4 ± 0.4	157 ± 15**	38.3 ± 3.7**#
2	0.8 ± 0.3	4.2 ± 0.2	60 ± 4	3.3 ± 0.9	4.7 ± 2.0**	4.8 ± 0.7	166 ± 13**†	38.6 ± 3.7**#
3	0.7 ± 0.2	4.2 ± 0.3	61 ± 4	3.3 ± 1.1	5.0 ± 1.7**	4.7 ± 1.2	171 ± 13**†	39.9 ± 5.1**#
4	0.9 ± 0.6	3.9 ± 0.7	62 ± 5	3.6 ± 0.5	5.5 ± 1.5**	4.8 ± 0.8	170 ± 11**†	39.7 ± 3.9**#

Block numbers 1 to 4 equate to 15 min bouts of intermittent exercise or rest. ** denotes a significant difference to the corresponding block in the resting trial ($P < 0.01$). # denotes a significant difference to the rest value in the exercise trial ($P < 0.01$). † denotes a significant difference to block 1 in the exercise trial ($P < 0.01$). Values are means ± S.D. (n=8).

Leukocyte Changes

Exercise-induced changes to the blood leukocyte populations increased immediately after the IEP, peaking at 2 h post-exercise, with the largest change occurring in the neutrophil population (Figure 5.4). Total leukocyte count 2 h after the 60 min trial period was $9.2 \pm 2.0 \times 10^9 \cdot l^{-1}$ cells in the exercise trial compared to $4.6 \pm 1.2 \times 10^9 \cdot l^{-1}$ cells in the rest trial (ES = 4.74, $P = 0.000$). This remained elevated for 6 h returning to resting values 24 h post-exercise. An increase in the monocyte population at 2 h post-exercise (ES = 1.87, $P = 0.006$) was accompanied by a decline in lymphocytes (ES = -1.11, $P = 0.047$) when compared to the corresponding time in the rest trial. The cell counts did not change significantly for any leukocyte subpopulation in the rest trial.

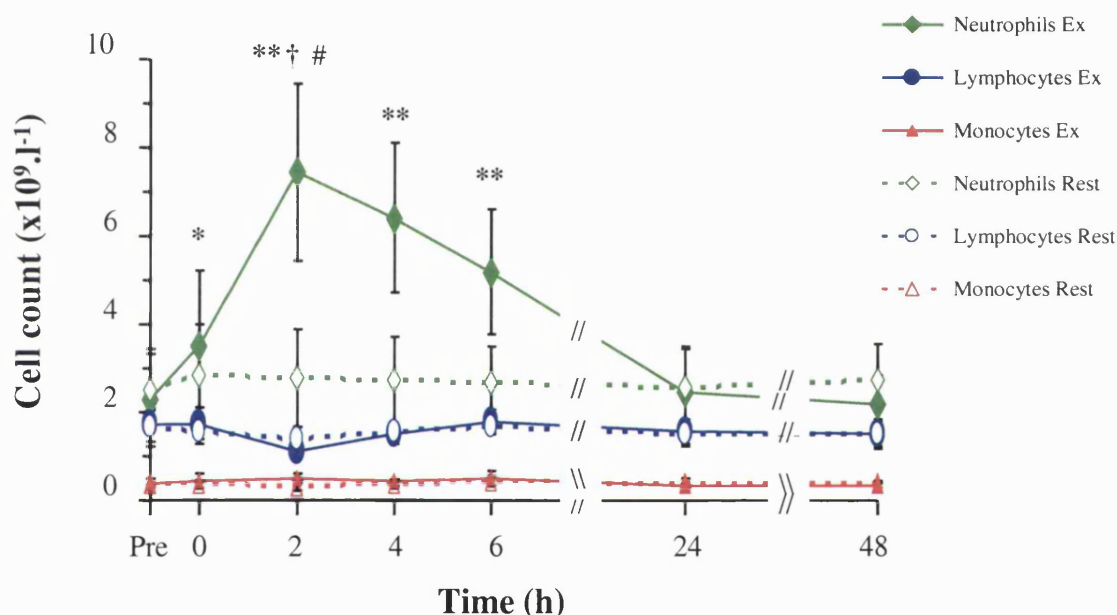


Figure 5.4 Neutrophil, lymphocyte and monocyte cell count ($\times 10^9 \cdot l^{-1}$) during the exercise and rest trial. Pre: pre-trial. ** denotes a significant difference between the change score in neutrophil cell count in the exercise trial compared to the change score at the same time point in the rest trial [ChangeEx minus ChangeRest] ($P < 0.01$), * ($P < 0.05$). † denotes a significant difference in the change score in the monocyte population in the exercise trial compared to the change score at the same time point in the rest trial ($P < 0.01$), # denotes a significant difference in the change score in the lymphocyte population in the exercise trial compared to the change score at the same time point in the rest trial ($P < 0.05$). Values are mean \pm S.D. (n=8).

Immunophenotyping of lymphocyte subpopulations CD3/4 and CD3/8 revealed no differences in distribution following exercise (Table 5.3) or with the time of day in the rest trial. The distribution of T cells remained between 73% and 75% over the 48 h period in both trials with B cell proportion remaining consistently between 21% and 24%.

Table 5.3 Immunophenotyping of lymphocyte subpopulations following 60 min intermittent exercise or rest

Trial	Time (h)						
	Baseline	0	2	4	6	24	48
Rest							
CD3/CD4	41.3 ± 7.0	45.6 ± 6.1	45.0 ± 5.5	47.6 ± 4.5	42.1 ± 6.6	45.0 ± 4.5	43.1 ± 6.8
CD3/CD8	29.7 ± 9.8	26.4 ± 3.8	28.3 ± 7.3	28.3 ± 4.4	28.3 ± 5.4	27.3 ± 3.6	27.6 ± 3.8
T cells	76.0 ± 7.9	73.7 ± 3.5	73.6 ± 3.7	74.5 ± 4.7	74.4 ± 2.2	74.1 ± 4.2	74.3 ± 5.3
B cells	23.0 ± 6.0	23.0 ± 3.3	23.5 ± 2.9	22.6 ± 3.6	23.0 ± 2.3	23.8 ± 1.9	23.3 ± 3.9
Exercise							
CD3/CD4	45.0 ± 5.2	45.4 ± 2.8	46.1 ± 3.3	45.6 ± 7.4	46.2 ± 5.1	45.1 ± 4.6	44.7 ± 5.9
CD3/CD8	28.0 ± 5.3	25.9 ± 3.4	26.0 ± 2.5	25.7 ± 2.5	28.1 ± 5.3	26.5 ± 3.3	28.5 ± 6.3
T cells	73.6 ± 5.6	73.6 ± 5.1	73.5 ± 5.3	76.9 ± 2.2	74.0 ± 4.1	75.1 ± 4.8	74.7 ± 2.4
B cells	23.6 ± 5.8	23.3 ± 4.4	23.8 ± 6.2	21.0 ± 3.0	23.5 ± 3.6	23.0 ± 5.9	23.0 ± 2.3

Values are percentages, expressed as mean ± S.D. (n=8).

HO-1 mRNA expression

The change in lymphocyte HO-1 mRNA following exercise was variable between subjects, with increases in HO-1 mRNA induction observed in some subjects and a lack of response in others (Figure 5.5). At 2 h post-exercise, four individuals had increased levels of HO-1 mRNA above 2-fold. The largest induction found was a 5-fold change at 2 h following exercise when compared to the corresponding time point in the control trial. Although the difference in HO-1 mRNA expression following exercise compared to rest was not statistically significant at any time point, an ES of 0.9 was observed at the 2 h post-trial time point indicating a large effect. The probability that the observed effect was at least as great as an ES of 0.2 was 75% at 2 h and 24 h post-trial (Figure 5.6).

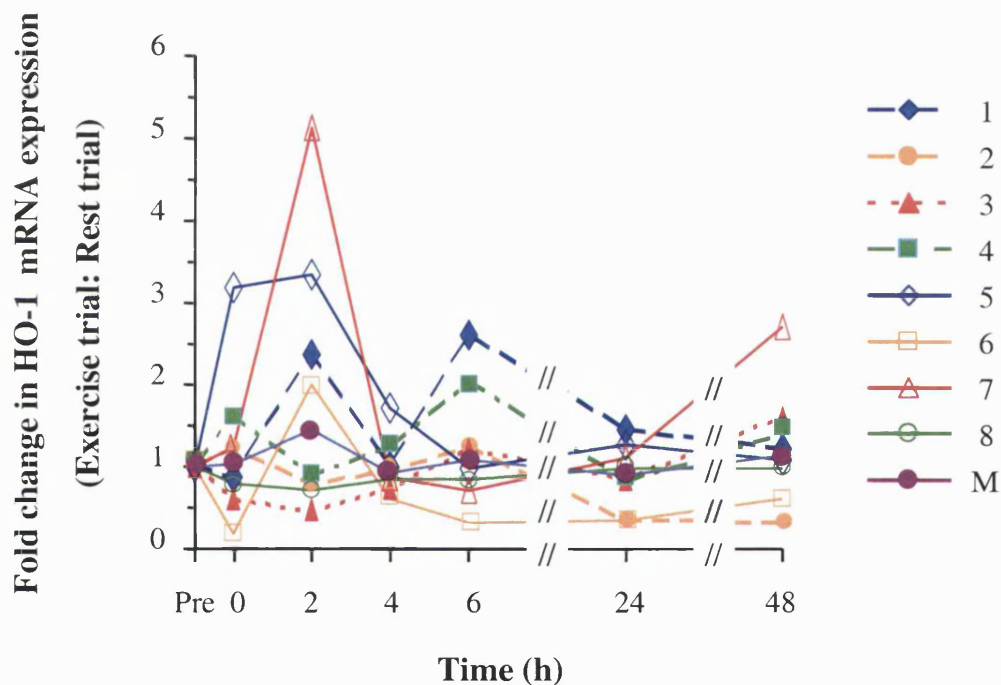


Figure 5.5 The fold change in lymphocyte HO-1 mRNA expression pre-exercise, immediately post-exercise, 2, 4, 6, 24, and 48 h after exercise in eight individuals. HO-1 mRNA was normalised to the baseline value in each trial and expressed as the fold change in HO-1 mRNA in the exercising trial relative to the fold change in HO-1 mRNA at the corresponding time point in the rest trial. Pre: pre-trial. M: median.

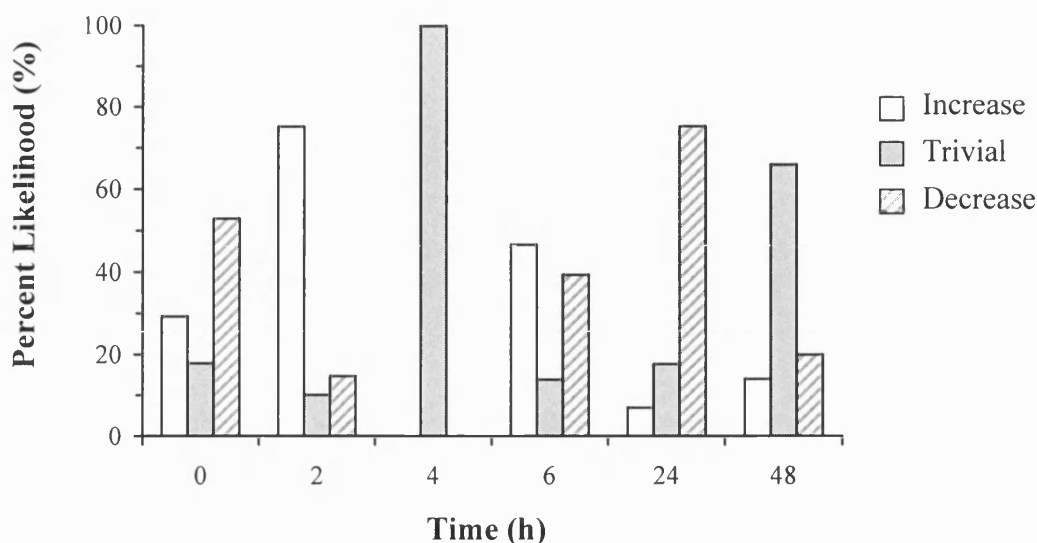


Figure 5.6 Estimates of the probability that the true values of the difference in the mean lymphocyte HO-1 mRNA expression (ChangeEx minus ChangeRest) over time are important. The smallest worthwhile effect was set *a priori* at an ES of 0.2. (n=8).

Where the percent likelihood is referred to as an increase, a greater change in HO-1 mRNA in the exercise trial compared to the change in HO-1 mRNA expression in the resting trial is observed. Therefore, at 2 h post-exercise it is 75% likely that the increase in HO-1 mRNA with exercise is greater than the change in HO-1 mRNA with rest. In contrast, at 24 h, the decrease indicates the change in HO-1 mRNA with exercise is less than the change in HO-1 mRNA observed at rest, indicating a down-regulation in HO-1 mRNA at this time point. The trivial effect at 4 h represents no difference between the change in HO-1 mRNA in either trial.

The basal level of HO-1 mRNA in lymphocytes was determined in each subject in both trials (Figure 5.7). In seven out of the eight subjects, HO-1 mRNA expression was higher in the exercise trial than in the rest trial. When this data was grouped, a significantly higher level of HO-1 mRNA at baseline was evident in the exercise trial with a value of 7.2 ± 2.1 arbitrary units (A.u) compared to 5.6 ± 2.0 A.u in the rest trial (mean \pm S.D., n=8) (ES = -0.8, $P = 0.043$). Trial order was randomised and there were no significant differences dependant on which trial was completed first.

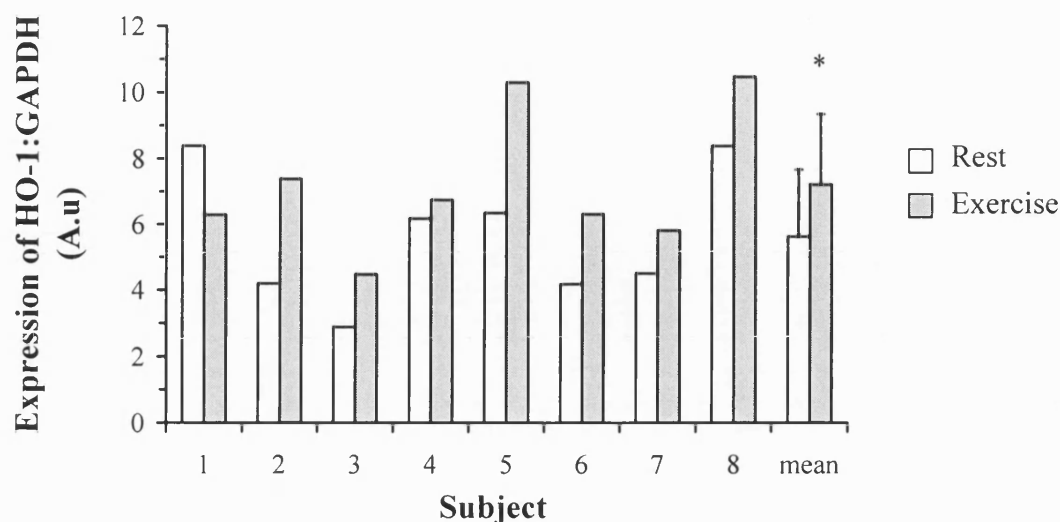


Figure 5.7 Basal expression of lymphocyte HO-1 mRNA in the exercise and rest trial in eight individuals including the mean \pm S.D. HO-1 mRNA is expressed relative to GAPDH in arbitrary units (A.u). * denotes a significant difference compared to the rest trial ($P < 0.05$).

The basal level of HO-1 mRNA expression was not related to the peak fold increase in HO-1 mRNA following acute intermittent exercise ($r = -0.33$, $P = 0.425$).

HO-1 Protein

HO-1 protein did not change with exercise or rest (Table 5.4). A moderate decrease in monocyte HO-1 protein following exercise was observed at 2 h post-exercise when compared to the rest trial (ES = -0.7, $P = 0.126$). This was associated with an 87% chance (decrease) that the true value was greater than the smallest worthwhile effect indicating the reduction in monocyte HO-1 protein following exercise compared to rest.

Table 5.4 The fold change in HO-1 protein expression at baseline, 0, 2, 4, 6, 24 and 48 h following 60 min exercise or rest

Trial	Time (h)						
	Baseline	0	2	4	6	24	48
Lymphocytes							
Rest	1.00 ± 0.00	0.94 ± 0.13	0.98 ± 0.14	0.97 ± 0.09	0.99 ± 0.20	0.95 ± 0.10	1.01 ± 0.25
Exercise	1.00 ± 0.00	1.01 ± 0.19	0.97 ± 0.25	0.95 ± 0.24	1.02 ± 0.23	1.01 ± 0.21	1.00 ± 0.15
Monocytes							
Rest	1.00 ± 0.00	1.16 ± 0.40	1.18 ± 0.33	1.14 ± 0.45	1.17 ± 0.67	1.14 ± 0.32	1.28 ± 0.74
Exercise	1.00 ± 0.00	1.05 ± 0.30	1.03 ± 0.38	1.02 ± 0.29	1.09 ± 0.41	1.18 ± 0.35	1.16 ± 0.28

Values are mean ± S.D. (n=8).

Hydrogen peroxide treatment

At the 6 h recovery time point following H₂O₂ treatment, there were no differences between lymphocyte or monocyte HO-1 protein induction following exercise or rest at any time. There were large effects in the difference in the mean at 0 h, 2 h, and 24 h post-exercise, suggesting a reduced lymphocyte HO-1 protein induction following H₂O₂ treatment when compared to the rest trial (Table 5.5). In monocytes, a large ES was observed 2 h post-exercise. Exercise resulted in a decreased monocyte HO-1 protein expression when compared with rest.

Table 5.5 The induction of HO-1 protein in human lymphocytes after exercise or rest following 6 h and 48 h recovery from H₂O₂ treatment

Time post-exercise (h)							
Trial	Baseline	0	2	4	6	24	48
6 h							
Rest	1.15 ± 0.29	1.28 ± 0.10	1.17 ± 0.24	1.10 ± 0.20	1.43 ± 0.31	1.43 ± 0.23	1.46 ± 0.25
Exercise	1.30 ± 0.27	1.29 ± 0.32	1.14 ± 0.33	1.21 ± 0.30	1.45 ± 0.26	1.34 ± 0.36	1.49 ± 0.34
Change		-0.14 ± 0.30*	0.27 ± 0.46*	-0.03 ± 0.32	-0.13 ± 0.38	-0.23 ± 0.42*	-0.11 ± 0.34
48 h							
Rest	1.36 ± 0.28	1.24 ± 0.61	1.78 ± 0.33	1.43 ± 0.39	1.88 ± 0.62	1.77 ± 0.73	1.62 ± 0.61
Exercise	1.54 ± 0.57	1.24 ± 0.54	1.25 ± 0.34	1.49 ± 0.40	1.53 ± 0.32	1.90 ± 0.83	1.57 ± 0.94
Change		-0.17 ± 0.46	-0.71 ± 0.62*#	-0.11 ± 0.43	-0.53 ± 0.54	-0.05 ± 0.61	-0.22 ± 0.63

Values are mean ± S.D. The symbols for ES and *P* values indicate significance of the change score relative to baseline [Change = (2 h post-exercise minus baseline) minus (2 h post-rest minus baseline)]. * denotes a large ES of the change score. # denotes a significant difference between exercise and rest trials (*P* < 0.05). (n = 8 for 6 h recovery, n = 7 for 48 h recovery).

At 2 h post-exercise, the expression of HO-1 48 h after recovery from H_2O_2 was significantly lower than the HO-1 protein induction at the same time point in the rest trial ($ES = -2.6$, $P = 0.044$) (Figure 5.8). In monocytes, a moderate effect was seen at the same time point, although this was not statistically significant ($ES = -0.7$, $P = 0.117$).

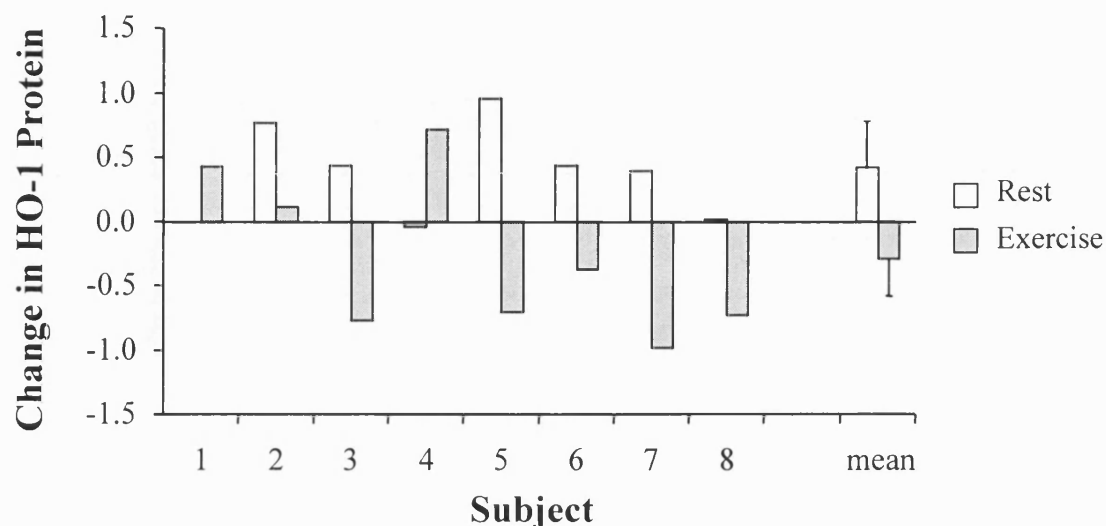


Figure 5.8 The individual change in lymphocyte HO-1 protein 48 h after H_2O_2 treatment 2 h post-exercise or 2 h post-rest (2 h post-exercise minus pre-exercise or 2 h post-rest minus pre-rest). The mean \pm S.D is included ($n=7$ for rest; $n=8$ for exercise). There was a missing value for subject one's rest trial as a result of a problem with the pre-rest treatment.

Likelihoods of the probability that the true value was greater than the smallest worthwhile effect were calculated for lymphocytes and monocytes at both 6 h and 48 h after H_2O_2 treatment, showing that the difference in the mean between exercise and rest trials were likely to decrease rather than increase (Figure 5.9 and 5.10).

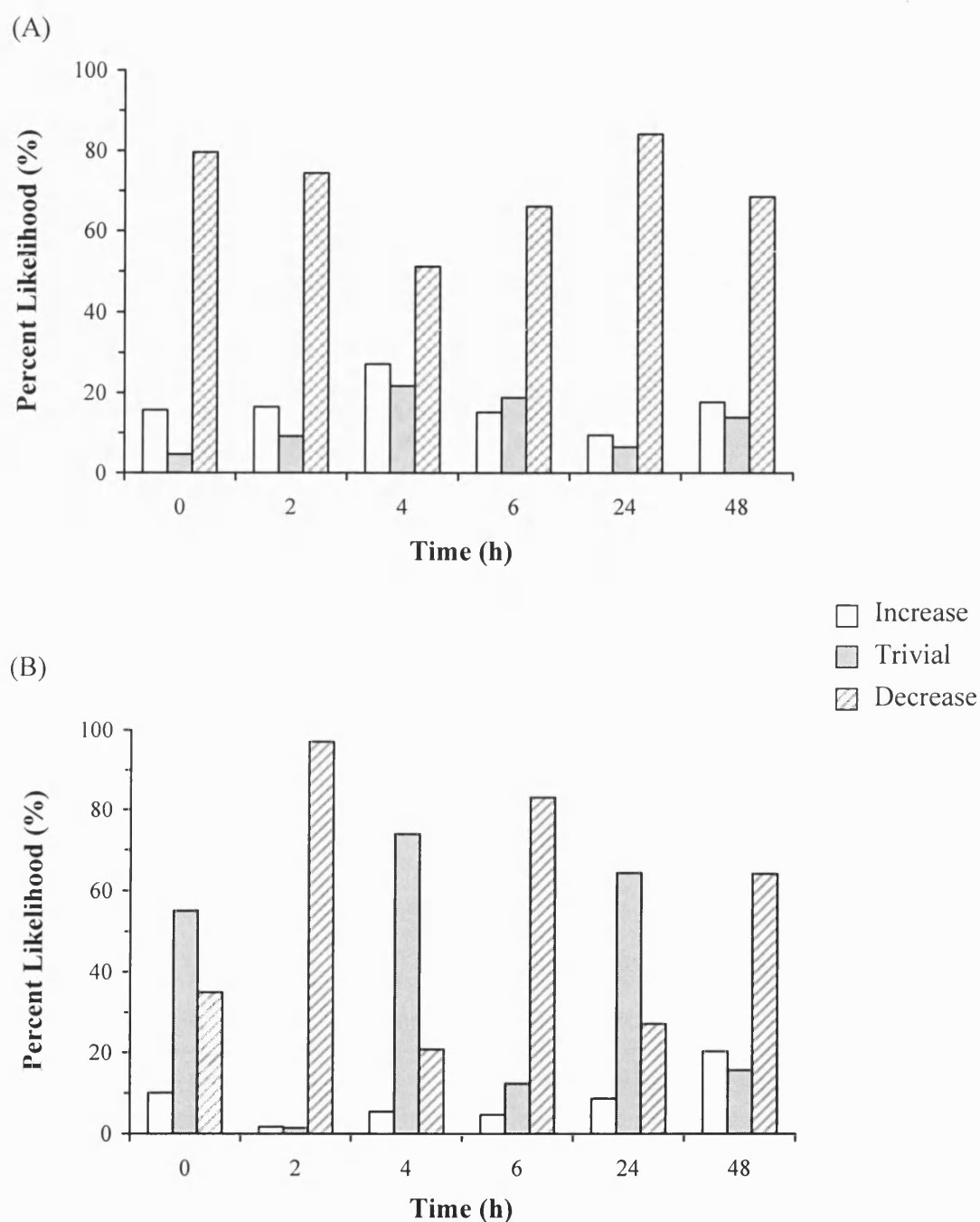
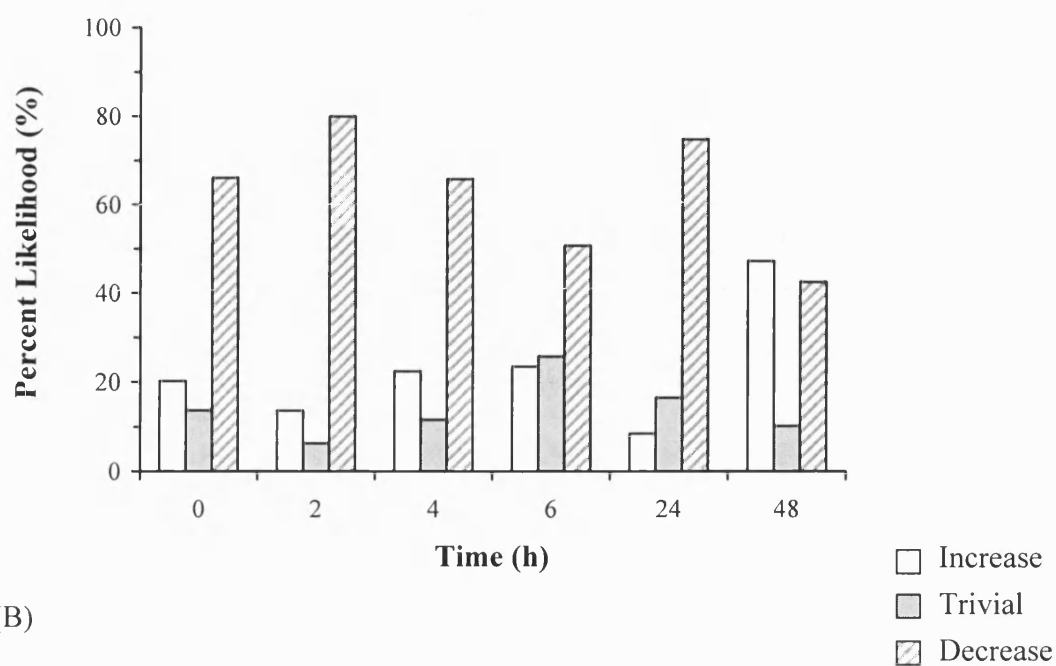


Figure 5.9 Estimates of the probability that the true values of the difference in the mean for lymphocyte HO-1 protein expression (ChangeEx minus ChangeRest) over time are important at 6 h recovery (A) and 48 h recovery (B). The smallest worthwhile effect was set at an ES of 0.2.

(A)



(B)

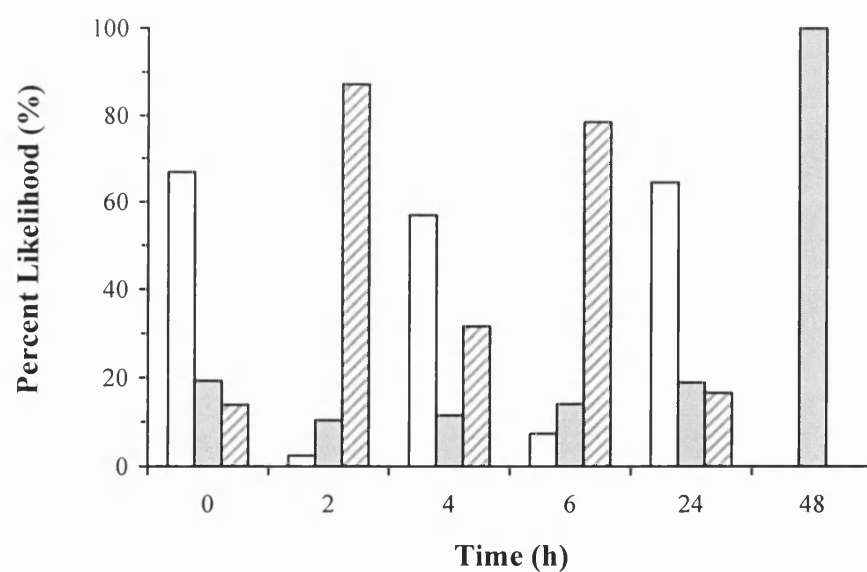


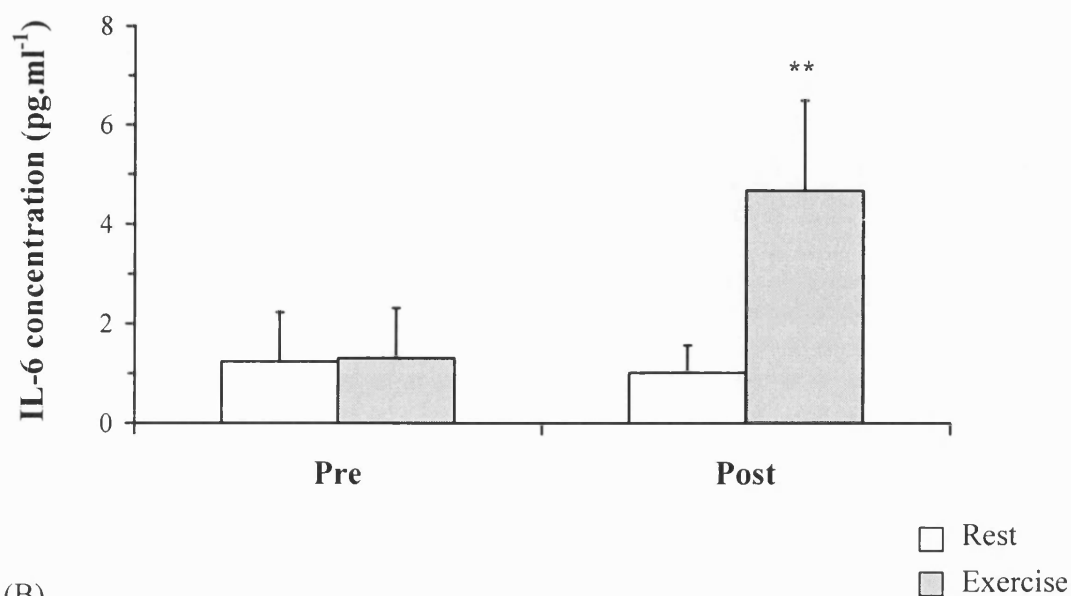
Figure 5.10 Estimates of the probability that the true values of the difference in the mean for monocyte HO-1 protein expression (ChangeEx minus ChangeRest) over time are important at 6 h recovery (A) and 48 h recovery (B). The smallest worthwhile effect was set at an ES of 0.2.

Cytokines

Plasma levels of interleukin-6 (IL-6) and interleukin-10 (IL-10) increased immediately following the exercise trial (Figure 5.11). There were no differences between IL-6 or IL-10 concentrations in the rest trial ($ES = -0.22$, $P = 0.310$ and $ES = 0.02$, $P = 0.697$ for IL-6 and IL-10, respectively). The difference in the change in the mean (ChangeEx minus ChangeRest) was significant for both plasma IL-6 and IL-10 concentrations ($ES = 2.44$, $P = 0.000$ and $ES = 2.03$, $P = 0.002$ for IL-6 and IL-10, respectively).

There was a positive relationship between the peak fold increase in plasma IL-6 concentration and peak fold increase in HO-1 mRNA expression in the exercise trial ($r = 0.723$, $P = 0.045$) (Figure 5.12). The peak fold change in plasma IL-10 concentration and peak fold increase in HO-1 mRNA expression in the exercise trial displayed a similar relationship, although was not statistically significant ($r = 0.577$, $P = 0.134$) (Figure 5.12).

(A)



(B)

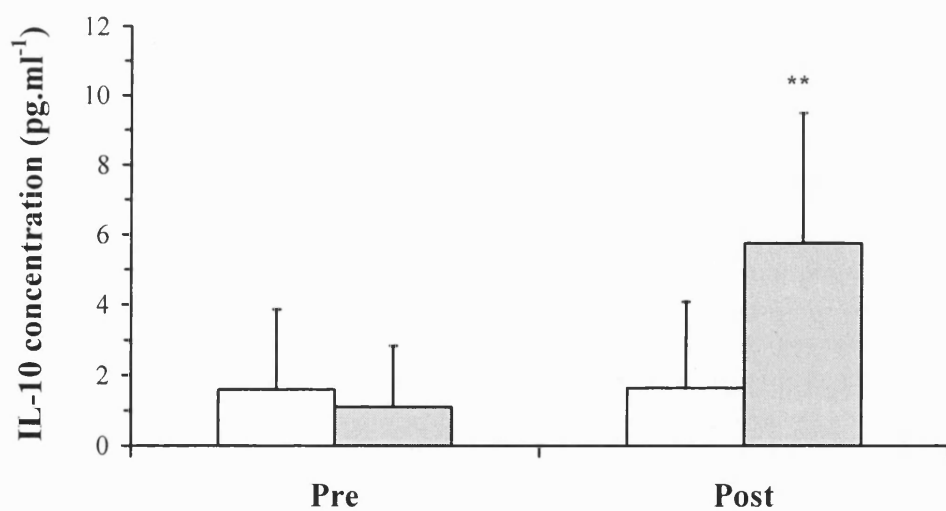


Figure 5.11 Plasma interleukin-6 concentrations (A) and interleukin-10 concentrations (B) in rest and exercise trials. Pre: pre-trial, Post: 0 h post-trial. ** denotes a significant difference between pre and post in the exercise trial ($P < 0.01$). Values are mean \pm S.D. (n=8).

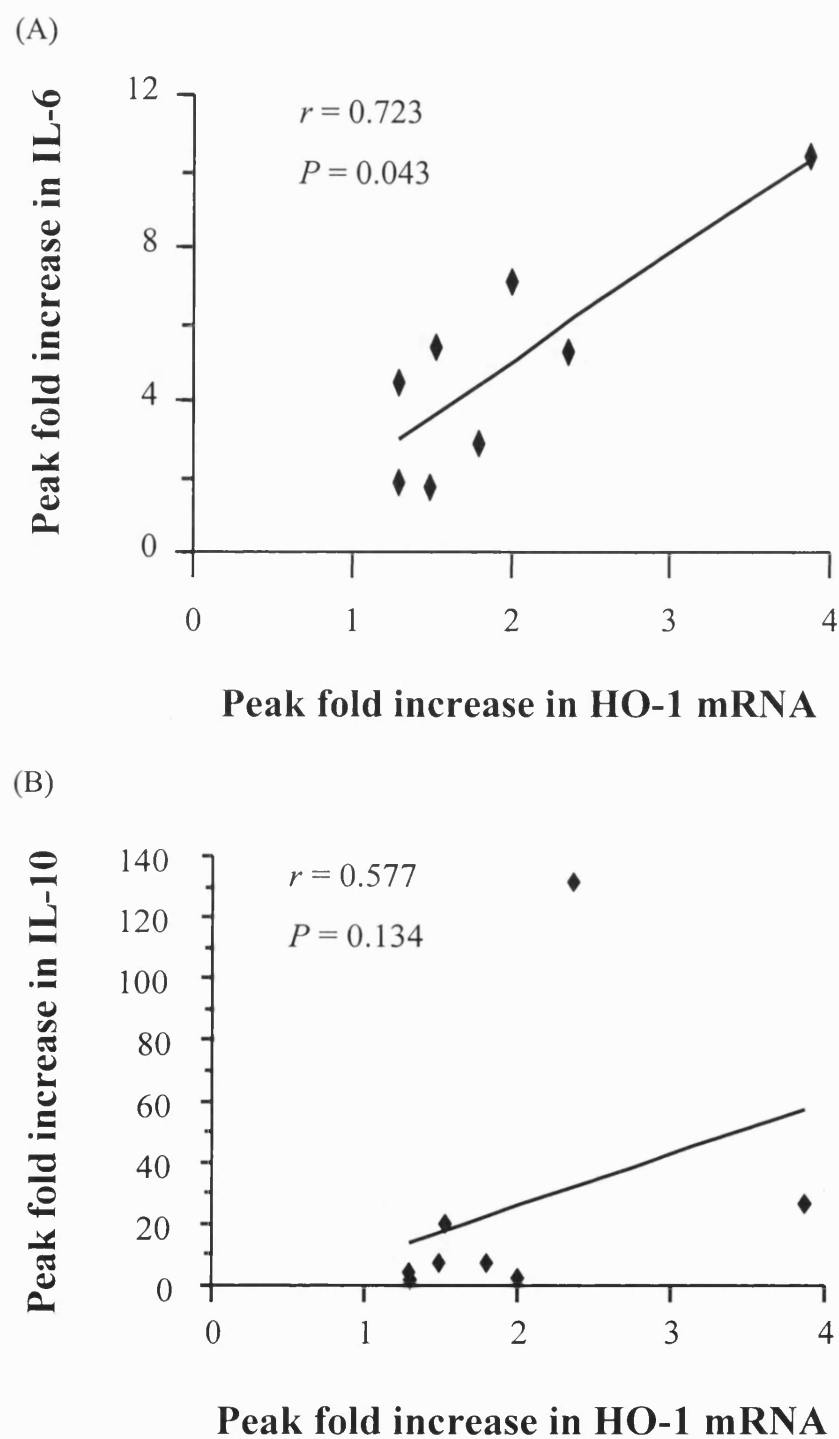


Figure 5.12 The relationship between the peak increase in HO-1 mRNA expression in mononuclear cells and the peak fold increase in plasma IL-6 concentration (A) and IL-10 concentration (B) following the intermittent exercise protocol. (n=8).

Cell Viability

Cell viability immediately following the isolation procedure was $97 \pm 2\%$ (mean \pm S.D.). Cell viability did not change with time or trial. Following H_2O_2 treatment, significant differences were observed between the sham-controls and H_2O_2 -treated samples at all time points in both the exercise and rest trials, for 6 and 48 h recovery ($P < 0.01$) (Table 5.6). An ES less than 0.2 was found when the difference in ChangeEx minus ChangeRest was calculated at all time points, therefore cell viability was unaffected by prior exercise.

Table 5.6 Cell viability (%) following recovery at 6 h and 48 h after H_2O_2 treatment, assessed over a time course post-exercise and post-rest

		Time post-rest or exercise (h)					
	Baseline	0	2	4	6	24	48
Rest (6 h following H_2O_2 treatment)							
Sham	94 ± 4	94 ± 4	93 ± 3	94 ± 3	93 ± 3	92 ± 3	94 ± 2
H_2O_2	$84 \pm 5^{**}$	$81 \pm 5^{**}$	$80 \pm 5^{**}$	$79 \pm 4^{**}$	$81 \pm 5^{**}$	$76 \pm 8^{**}$	$81 \pm 8^{**}$
Exercise (6 h following H_2O_2 treatment)							
Sham	93 ± 3	95 ± 2	95 ± 3	94 ± 3	94 ± 5	91 ± 5	91 ± 5
H_2O_2	$82 \pm 8^{**}$	$82 \pm 7^{**}$	$82 \pm 7^{**}$	$80 \pm 5^{**}$	$81 \pm 7^{**}$	$79 \pm 7^{**}$	$75 \pm 9^{**}$
Rest (48 h following H_2O_2 treatment)							
Sham	83 ± 5	83 ± 6	82 ± 6	78 ± 7	81 ± 6	79 ± 10	85 ± 7
H_2O_2	$60 \pm 3^{**}$	$58 \pm 6^{**}$	$57 \pm 5^{**}$	$57 \pm 3^{**}$	$57 \pm 4^{**}$	$59 \pm 6^{**}$	$55 \pm 3^{**}$
Exercise (48 h following H_2O_2 treatment)							
Sham	80 ± 7	80 ± 6	78 ± 5	80 ± 6	81 ± 6	79 ± 5	81 ± 3
H_2O_2	$58 \pm 7^{**}$	$56 \pm 7^{**}$	$58 \pm 6^{**}$	$54 \pm 6^{**}$	$59 \pm 6^{**}$	$57 \pm 5^{**}$	$56 \pm 5^{**}$

** denotes a significant difference when compared to the sham-treated sample at the same respective time point ($P < 0.01$). Values are mean \pm S.D. (n=8).

5.4 Discussion

In this investigation, lymphocytes harvested 2 h after an acute bout of demanding exercise demonstrated a reduced up-regulation of HO-1 protein expression in response to a second oxidant challenge. This was also the time point where the increase in lymphocyte HO-1 mRNA was greatest. This is the first investigation that demonstrates altered lymphocyte HO-1 protein response to exogenous oxidant treatment following acute exercise.

The exercise protocol used in this study was intermittent in order to achieve a higher overall intensity of exercise. The increases in blood lactate, heart rate and oxygen consumption confirm the demanding nature of the exercise. Interestingly, the increase in total leukocytes and neutrophils following exercise was smaller than that observed following a half marathon (Chapter 3), which may reflect the shorter duration of exercise. Additionally, the proportion of T cells and B cells did not change post-exercise. The intensity of the protocol used in this study was equivalent to approximately 75% $\dot{V}O_{2\max}$. Although it was shorter in duration than the prolonged protocols previously shown to induce HO-1 (Niess *et al.*, 1999b; Thompson *et al.*, 2005), it was hypothesised that higher intensity bouts of exercise would increase the demands of the cardiovascular system associated with overall greater oxygen consumption. In particular, the present protocol involves subjects completing four bouts of sustained work at 100% maximal O_2 consumption. A single incremental maximal exercise test that terminates at an equivalent to 100% maximal O_2 consumption markedly increases free radical concentration in blood (Ashton *et al.*, 1998). During mitochondrial oxidative phosphorylation there is a reduction of molecular oxygen, with 2-10% oxygen undergoing incomplete reduction leading to the generation of superoxide (Halliwell and Gutteridge, 1999). Although still not fully resolved, it is generally accepted that the quantity of superoxide will increase in proportion to oxygen uptake. In the current investigation, an 11-fold increase in oxygen consumption above resting values was observed during the lowest intensity phases of the intermittent protocol.

Previous studies have demonstrated both prolonged and high-intensity exercise induces HO-1 mRNA in skeletal muscle (Pilegaard *et al.*, 2000) and leukocytes (Thompson *et al.*, 2005). In human skeletal muscle, levels of HO-1 mRNA were shown to increase 11-fold above control levels 2 h after an exhaustive one-legged knee extensor exercise equivalent to 70% of each individual's two min maximal resistance load (Pilegaard *et al.*, 2000). In comparison, a 4 h cycle at 60% $\dot{V}O_{2\max}$ resulted in only a 4-fold increase in HO-1 mRNA which remained elevated for 4 h (Pilegaard *et al.*, 2000). Therefore,

high-intensity eccentric type exercise is more effective in up-regulating HO-1 mRNA levels in skeletal muscle. The exercise protocol used in the present study involved high-intensity intermittent running as it was hypothesised this would induce a greater expression of HO-1 mRNA in lymphocytes than prolonged running. The intermittent exercise used in the current study increased endogenous HO-1 mRNA levels 2 h post-exercise when compared to the corresponding time point in the control trial. The expression of HO-1 mRNA did not change in the resting trial. There was a trend for an increase in lymphocyte HO-1 mRNA post-exercise as indicated by the large effect size and likelihood values. However, the induction of HO-1 mRNA was variable between individuals with some subjects showing a down-regulated response to exercise. The maximal induction of HO-1 mRNA in the current study was a 5-fold increase 2 h post-exercise with a total of five subjects having a 2-fold change in HO-1 mRNA between 0 and 6 h following exercise. The basal level of protective genes has been suggested to reflect the inducibility of the gene in response to stress (Boshoff et al, 2000), with low basal expression reflected by high induction and *vice versa*. In the present investigation, no relationship between basal HO-1 mRNA expression and the peak induction of HO-1 mRNA expression was evident ($r = -0.33$, $P = 0.425$). In the current study, individuals with either low or high basal expression of HO-1 mRNA were able to induce the gene.

Presently, an up-regulation in lymphocyte HO-1 mRNA has been demonstrated following prolonged exercise equivalent to 70% $\dot{V}O_{2\max}$ lasting 75 min (Thompson *et al.*, 2005). Although the protocol used in the current investigation was completed at a higher overall intensity than the prolonged running by Thompson and co-workers (2005), the duration was 15 min shorter and there was a total of six min rest from exercise. The exercise-induced increase in HO-1 mRNA observed by Thompson and colleagues (2005) was larger than the response to the intermittent protocol. Exhaustive exercise has been shown to alter intracellular calcium signalling of lymphocytes leading to changes in the mitochondrial membrane potential (Mooren *et al.*, 2001). It is possible that the rest periods in the exercise protocol were sufficient for calcium disturbances within the mitochondria to recover and, coupled with the re-synthesis of antioxidant compounds such as glutathione, result in an attenuation of overall oxidative stress. Therefore, it would appear that the HO-1 mRNA response in skeletal muscle and lymphocytes differ depending on the exercise mode and intensity with prolonged running the more effective protocol for up-regulating HO-1 mRNA in human lymphocytes.

Inflammatory processes play a role in the generation of free radicals and resulting oxidative stress. In the current study, an increase in leukocyte counts was observed with

the greatest changes to the neutrophil population at 2 h post-exercise. This increase in total leukocyte cell count was accompanied by an increase in monocyte cell number and a decline in lymphocyte cell number. Neutrophils and monocytes have the capability to produce superoxide, migrating to damaged muscles where they function to remove the injured tissue and aid the repair process (Pyne, 1994). The release of pro-inflammatory cytokines amplifies ROS formation and augments oxidative stress (Niess *et al.*, 1999a). In the present investigation, the plasma concentration of the pro-inflammatory cytokine IL-6 significantly increased immediately following exercise and the fold change in IL-6 was positively correlated with the peak fold increase in HO-1 mRNA expression after exercise. The anti-inflammatory cytokine IL-10 also increased following intermittent exercise. Increased plasma IL-6 concentration was observed following prolonged running (Thompson *et al.*, 2005), although the concentration of plasma IL-6 in this investigation was higher than the levels found in the current study. Previously, a correlation between IL-8 and HO-1 protein expression has been found in human lymphocytes and neutrophils following a half marathon race (Niess *et al.*, 1999b). Collectively, these findings support a possible relationship between pro-inflammatory cytokine concentration and HO-1 expression.

In contrast to expectations, the acute exercise protocol used in the present study did not lead to an increase in HO-1 protein expression. Furthermore, in monocytes there was a moderate reduction in HO-1 protein expression 2 h post-exercise. In contrast to the findings of the present study, other investigations have demonstrated increases in HO-1 protein expression post-exercise (Niess *et al.*, 1999b; Thompson *et al.*, 2005). The conflicting results observed in this study may be due to differences in the exercise protocols and/or methods of analysis. Flow cytometric analysis of MNCs in this investigation used the median fluorescence intensity to calculate fold changes in HO-1 protein expression. Where significant increases in HO-1 protein expression have been demonstrated with this method, percent positive cells and the mean fluorescence intensity have been the parameters used for analysis (Niess *et al.*, 1999b; Fehrenbach *et al.*, 2003a). Although this may partially explain the discrepancy in results, it is noteworthy that the exercise protocols employed in earlier investigations have also been longer in duration. Therefore, it is possible that the discrepancy in results regarding exercise-induced HO-1 protein may be due to the duration and intensity of the exercise protocols employed, with HO-1 protein increasing in response to prolonged continuous exercise and not to demanding intermittent exercise that incorporates rest periods.

Previously, HO-1 protein induction has been found to be maximal 48 h following treatment with H₂O₂ (Chapter 4). In the present study, lymphocyte HO-1 protein

induction increased approximately 1.5-fold 48 h following H_2O_2 treatment prior to exercising or resting for 60 min. Following the intermittent exercise, a decrease in the ability of lymphocytes to respond to H_2O_2 was evident as the induction of HO-1 protein was smaller when compared to pre-exercise values. The HO-1 protein response to H_2O_2 treatment is a reproducible measure (Chapter 4) and therefore the decrease in HO-1 protein observed 2 h post-exercise is the result of changes due to exercise. Increased HO-1 mRNA expression was found at this time point after exercise; although this was not accompanied by an increase in HO-1 protein at this point or any later time. The reduction in the HO-1 response to H_2O_2 treatment may reflect an inability to respond to subsequent oxidative stress. This could represent a maladaptive response of prior demanding exercise as lymphocytes were unable to induce the gene. However, it is noteworthy that this response was not accompanied by a decrease in cell viability. Furthermore, this was only a transient response and 4 h after exercise the ability to respond to H_2O_2 had returned to pre-exercise levels.

It is possible that the lack of induction of HO-1 to H_2O_2 treatment post-exercise is a reflection of the many other changes induced by an acute bout of exercise. Acute exercise is known to increase the expression of HSPs and antioxidant enzymes in lymphocytes (Fehrenbach *et al.*, 2000a; Elosua *et al.*, 2003; Tauler *et al.*, 2003). Therefore, one explanation for the lack of increase in HO-1 protein following *ex vivo* treatment may potentially be a consequence of increases in HSP and endogenous antioxidants and indicate an adaptive response to exercise. If this were subsequently proved to be the case this would potentially have important implications since lymphocytes are continuously exposed to ROS and are often found at sites of inflammation where further ROS are generated. Several investigations have shown an adaptive response to subsequent oxidative stress using a variety of initial stressors which induce HO-1 (Noel and Tyrrell, 1997; Speit *et al.*, 2000; Rothfuss and Speit, 2002; McArdle *et al.*, 2004). The time for this adaptive response has varied from 4 h (McArdle *et al.*, 2004) to 48 h following the initial exposure (Noel and Tyrrell, 1997), whereas in the present investigation the decrease in HO-1 induction following *ex vivo* treatment was observed 2 h post-exercise. The reduced ability of lymphocytes to induce HO-1 protein to subsequent oxidant challenge was a transient effect as the response had returned to resting levels 4 h following exercise. Furthermore, the reduced HO-1 response to H_2O_2 treatment 2 h following exercise was not mediated by HO-1 as the acute exercise employed in the present investigation did not increase HO-1 protein.

An alternative explanation for the lack of induction of HO-1 following prior exercise is explained by the concept of 'refractoriness'. Noel and Tyrrell (1997) investigated the

adaptive response to a second challenge of oxidative stress in skin fibroblasts using UVA irradiation and reported an absence of HO-1 mRNA accumulation with a treatment 48 h following the first irradiation. The down-regulated HO-1 response following prior induction has been described as refractoriness, and it is proposed that this may represent a pathway that has evolved to maintain haem synthesis (Noel and Tyrrell, 1997). This led to the proposal that a haem intermediate was involved in the refractory response (Noel and Tyrrell, 1997) with later investigations confirming this in the identification of the HO-1 repressor, the haem-binding protein Bach-1 (Shibahara *et al.*, 2003). When haem binds to Bach-1, the repressor function is prevented (Ogawa *et al.*, 2002) allowing induction of HO-1. The continuous re-induction of HO-1 would lead to local accumulation of CO, bilirubin and iron which individually are cytotoxic at high concentrations. The effects of unwanted gene activation was illustrated in cells where HO-1 is highly over-expressed, reversing the cyto-protective effects associated with increased HO-1 expression (Suttner and Dennery, 1999). The continuous re-induction of HO-1 would prevent the replacement of crucial haem-containing proteins and, therefore, repression of HO-1 could be a safety mechanism preventing unnecessary gene activation. An alternative proposal for the repression of HO-1 is that down-regulation of the gene might be a mechanism to reduce energy expenditure for haem catabolism and prevent accumulation of its by-products (Shibahara *et al.*, 2003). A final possibility is that the basal expression of HO-1 may have been adequate for the level of oxidative stress encountered during exercise, thus increased protection was not necessary. According to this explanation, the preconditioning stimulus in the present study may have been sufficient to modestly alter transcription but this was not sufficient to increase protein synthesis.

In summary, the findings from this investigation confirm that an acute bout of exercise induces HO-1 mRNA accumulation in lymphocytes. However, in contrast to expectations, the increase was generally modest and was not associated with an increase in HO-1 protein. The degree of these changes limits the scope of this particular form of exercise to serve as a model for subsequent modulation with lycopene supplementation. In spite of the modest change in HO-1, there was a decrease in the ability of lymphocytes to respond to H₂O₂ treatment following an acute bout of exercise. It is possible that this reflects either an adaptive response to prior oxidative stress (e.g., due to increases in antioxidant enzymes), refractoriness of HO-1 or some form of maladaptation and failure to respond when required.

CHAPTER 6

THE IMPACT OF LYCOPENE SUPPLEMENTATION VIA WHOLE FOODS ON MODULATION OF HO-1 EXPRESSION AND CELL VIABILITY IN HUMAN MONONUCLEAR CELLS

6.1 Introduction

Dietary supplementation with antioxidants can enhance the body's natural defence system and therefore has the potential to modify the response to oxidative stress. The carotenoid lycopene is a powerful free radical scavenger and there is a wealth of epidemiological evidence supporting a protective effect of lycopene against certain types of cancers (Gann *et al.*, 1999; Giovannucci, 1999; Lu *et al.*, 2001), atherosclerosis and cardiovascular disease (Kohlmeier *et al.*, 1997; Klipstein-Grobusch *et al.*, 2000; Rissanen *et al.*, 2003). However, investigations using dietary supplementation of this antioxidant have, like many other exogenous antioxidants, resulted in conflicting findings regarding a protective role for lycopene (Riso *et al.*, 1999; Astley *et al.*, 2004; Riso *et al.*, 2004).

Lycopene is the predominant antioxidant found in tomatoes and supplementation studies have often provided this carotenoid in the form of tomato-based products. The bioavailability of lycopene from processed tomato products is greater than that found in fresh tomatoes (Stahl and Sies, 1992; Gartner *et al.*, 1997; Stahl *et al.*, 2002). Collectively, the evidence supports a protective effect from lycopene supplementation in whole food products (Rao and Agarwal, 1998; Gann and Khachik, 2003).

In the previous chapter, the HO-1 mRNA response to an acute bout of exercise was inconsistent between subjects with an increase in HO-1 mRNA observed post-exercise in four individuals with the remaining four subjects experiencing a down-regulation in HO-1 mRNA post-exercise (Chapter 5). This finding makes the exercise and combined *ex vivo* model inappropriate to use in further investigations in conjunction with lycopene supplementation. Since lymphocyte treatment with H₂O₂ *ex vivo* up-regulated HO-1 protein in all individuals (Chapter 4) this represents a more appropriate model for intervention than the previous exercise model. In addition, several investigations have demonstrated that human lymphocytes treated *ex vivo* with H₂O₂ induces DNA damage

(Marini *et al.*, 1996b; Rothfuss *et al.*, 1998) and cell death by apoptosis (Marini *et al.*, 1996a; Fenech *et al.*, 1999) and that antioxidant supplementation modulates this response. Indeed, antioxidant treatment prior to H₂O₂ exposure *in vitro* has been shown to reduce both apoptotic cell death and DNA damage (Crott and Fenech, 1999; Lowe *et al.*, 1999). Furthermore, lycopene supplementation via a range of tomato products reduces DNA damage following H₂O₂ treatment *ex vivo* (Porrini and Riso, 2000; Porrini *et al.*, 2005). The effect of lycopene supplementation *in vivo* and the apoptotic response of lymphocytes to *ex vivo* H₂O₂ treatment have not previously been examined. Although findings from cell culture and animal studies suggest that HO-1 induction would be suppressed, further evidence regarding the effect of antioxidant supplementation and modulation of HO-1 gene expression and the mode of cell death *ex vivo* are required.

Physical activity recommendations suggest that thirty minutes of moderate activity on five or more days of the week is sufficient to maintain health (Pate *et al.*, 1995). Because habitual physical activity has the capacity to modulate endogenous antioxidants and therefore the response to oxidative stress, physical activity assessment should be incorporated to explore the role of habitual physical activity in HO-1 induction and cell death. Therefore, the primary aim of the present study is to investigate the impact of lycopene, using tomato supplementation, on modulation of the HO-1 response to oxidative stress and mode of cell death in response to H₂O₂ treatment. A secondary aim is to examine the relationship between these parameters and physical activity in order to (i) examine whether individuals participating in regular physical activity had different responses and (ii) whether physical activity *per se* is important in explaining inter-individual differences.

6.2 Methods

6.2.1 Subjects

Twenty-four male volunteers took part in this study (Table 6.1). All participants were non-smokers and not taking regular prescribed medication. Participants with a range of physical activity levels were recruited.

Table 6.1 Subject characteristics

Age (yrs)	Height (cm)	Mass (kg)	Σ Skinfolds (mm)
26.3 \pm 4.0	180 \pm 10	76.9 \pm 10.8	36 \pm 12

Values are mean \pm S.D. (n=24).

6.2.2 Experimental design and procedures

Anthropometric data was collected on the first visit (Chapter 2.2) and physical activity was assessed during the wash-out period (Section 6.2.5). During this investigation, participants were asked to refrain from consuming foods containing lycopene for eight-weeks (Appendix 3). The study design consisted of two, three-week supplementation periods separated by a two-week wash-out period (Figure 6.1). The supplementation periods consisted of (a) 170 g passata sauce (containing approximately 7 mg lycopene and 0.3 mg β -carotene) (H.J. Heinz, UK) taken with 10 g butter (Anchor, Arla foods, UK) or (b) 10 g butter alone. The tomato product was consumed with butter to ensure there was no loss in the bioavailability of lycopene from ingestion to absorption (Stahl and Sies, 1992; Van het Hof *et al.*, 2000; Stahl *et al.*, 2001) and was obtained from the same batch of tomatoes. A cross-over design was used with subjects acting as their own control, therefore in the control condition, no tomato products were consumed. The order of supplementation was randomised.

Subjects were asked to maintain a food and fluid record for three days of each week during the supplementation periods (Chapter 2.4). These days were any three days out of seven and were used as a tool by the experimenter to monitor adherence to the intervention. In the final week leading up to the next blood sample, subjects were instructed to report the three days immediately prior to the sample and were then asked to replicate these foods prior to visit five (Table 6.2). During the wash-out time, subjects did not weigh their foods and maintained a near to normal diet, still avoiding foods containing lycopene.

Table 6.2 Daily dietary composition assessed over three days prior to each trial day

Energy Intake (kcal)	CHO (%)	Protein (%)	Fat (%)	Vitamin C (mg)	Vitamin E (mg)	β -carotene (mg)
2619 \pm 791	49 \pm 6	17 \pm 6	34 \pm 8	183 \pm 273	8 \pm 3	1 \pm 1.4

In the tomato supplemented trial, lycopene intake was $\sim 7 \text{ mg}\cdot\text{day}^{-1}$. The lycopene content of foods was determined using an updated database of U.S foods (Holden *et al.*, 1999). CHO: carbohydrate. Values are mean \pm S.D. (n=24).

Subjects reported to the laboratory following an overnight fast, on five occasions (Figure 6.1). A resting blood sample (10-50 ml) was taken from a forearm vein following 15 min in a supine position (Chapter 2.6). On visits one, two, and four, 10 ml of venous blood was collected. For visits three and five (at the end of each three-week supplementation period) 25-50 ml blood was collected to obtain a final yield of a minimum of approximately 40×10^6 MNCs. Plasma was collected for carotenoid analysis (Section 6.2.4) at each occasion and stored in aliquots at -70°C . Mononuclear cells were isolated (Chapter 2.6.1) and basal values for HO-1 protein (Chapter 2.9) and determination of percent apoptosis and necrosis (Section 6.2.3) were taken on four of the five visits. Following visits three and five, MNC's were treated with H_2O_2 (Chapter 2.8.1) and levels of HO-1 protein, apoptosis and necrosis were determined at 18 and 48 h following the initial treatment. Previous work showed similar levels of apoptosis in freshly harvested lymphocytes at 6, 12 and 18 h following treatment with $50 \mu\text{M}$ H_2O_2 (Figure A.4, Appendix 8). Therefore, the 18 h time point was chosen as the induction of HO-1 is delayed. The 48 h time point corresponds to the maximal induction of HO-1 protein (Chapter 4).

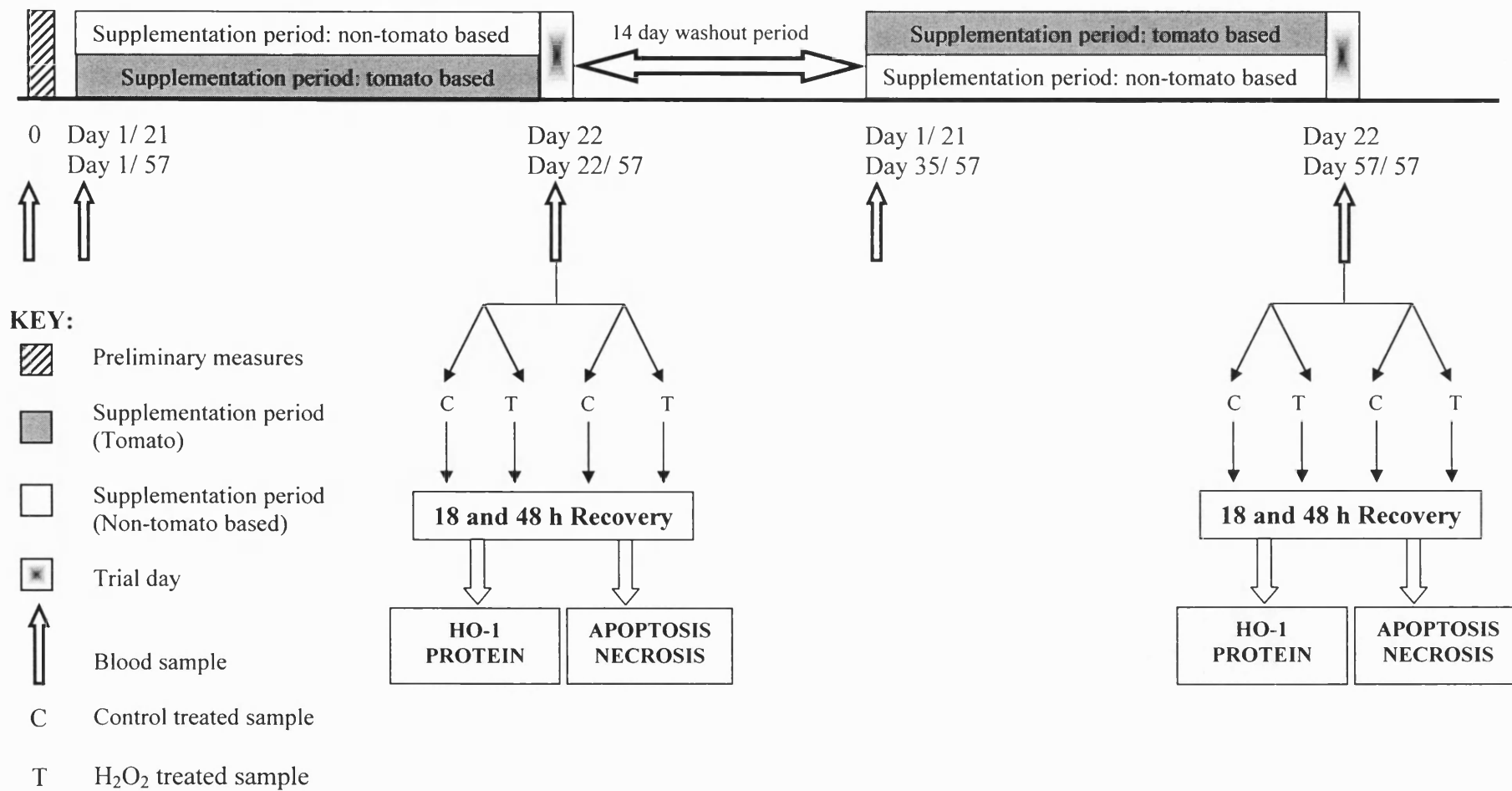


Figure 6.1 Experimental design of the tomato supplementation vs. no tomato condition. Each subject acts as their own control with the order of supplementation randomised. Each supplementation period lasts three weeks and is separated by a two week wash-out phase.

6.2.3 Determination of apoptosis and necrosis

The use of flow cytometry allows for the quantification of live, apoptotic and necrotic cells on a single-cell basis within cell populations. The combination of a specific marker of apoptosis and a DNA stain can be used to distinguish between apoptotic and necrotic cell death. Markers of apoptosis and necrosis were FITC-conjugated Annexin V (AV) and the DNA stain propidium iodide (PI). Annexin V is a Ca^{2+} -dependent phospholipid binding protein with a high affinity for phosphatidylserine (PS). It stains specifically externalized PS in the plasma membrane of apoptotic cells (Vermes *et al.*, 2000). As necrotic cells also expose PS, these cells are distinguished by simultaneous staining with PI.

Cells were stained with AV and PI as described in the manufacturer's protocol (Roche Applied Science, Lewes, UK). Briefly, MNCs were washed with PBS following incubation and resuspended in 100 μl incubation buffer (10 mM Hepes/NaOH, pH 7.4, 5 M NaCl, 100 mM CaCl_2) containing AV (20 $\mu\text{l}.\text{ml}^{-1}$) and PI (20 $\mu\text{l}.\text{ml}^{-1}$). Cells were incubated in the dark for 15 min at room temperature. Following this, 400 μl incubation buffer was added to each sample before analysis in a Becton Dickinson FACScan (Cellquest version 3.3 software, Belgium). Samples were analysed using 488 nm excitation and detection in the FL1 channel for AV (530 \pm 30 nm bandpass filter for fluorescein) and FL3 channel (650 nm longpass filter) for PI, using electronic compensation to correct for spectral overlap. The data were analysed using two-dimensional dot plots of FL3 *versus* FL1 fluorescent profiles (Figure 6.2). Live (AV⁻/PI⁻), apoptotic (AV⁺/PI⁻) and necrotic (total PI⁺) cell populations were determined in a total of 10,000 cells and expressed as a percent. The apoptotic and necrotic cells in sham-treated samples were subtracted from the corresponding populations in H_2O_2 -treated samples to exclude the low levels of death unrelated to H_2O_2 .

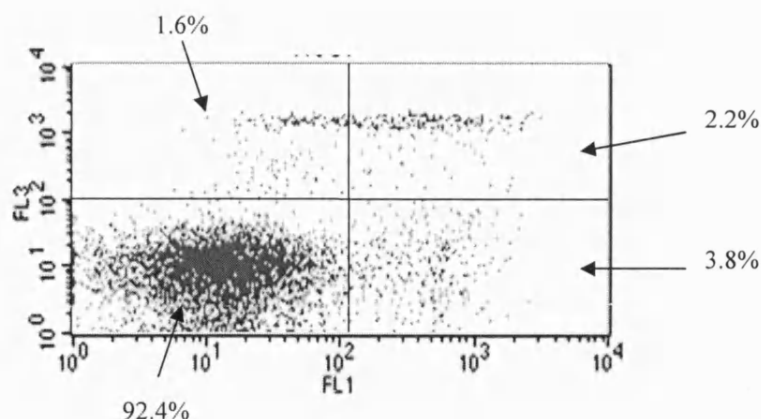


Figure 6.2 Dual parameter dot plot of FL3 *versus* FL1 fluorescent profiles in an untreated sample. Live cells are located in the lower left quadrant (AV⁻/PI⁻), with apoptotic cells in the lower right quadrant (AV⁺/PI⁻). The upper left and right quadrants reflect necrotic cell death (total PI⁺).

6.2.4 Detection of lycopene

Human plasma was thawed and vortexed to disperse possible precipitates. In 5 ml tubes, 250 μ l of plasma was added to 250 μ l distilled water, followed by 500 μ l ethanol containing butylated hydroxytoluene (BHT) (0.2g.l⁻¹) to deproteinise the sample. The suspension was mixed for approximately 20 s on a vortex mixer. The extraction was completed by adding 2 ml *n*-hexane, and samples were mixed for 90 s prior to centrifugation (2500 g, 5 min at room temperature). The upper phase was extracted into a glass tube and dried in a N₂ current. The residue was re-dissolved in 250 μ l ethanol (containing BHT) and placed in aliquots in glass amber vials prior to analysis by HPLC.

High-performance liquid chromatography was performed using a modified protocol described by Tauler and colleagues (2002). The HPLC system consisted of a pump (JASCO PU-1580, Japan), an ultraviolet/visible light detector (JASCO UV-1575, Japan), and an autosampler (JASCO 851-AS, Japan), coupled to an integrator (Hewlett-Packard, model 3395) with a C18, 4.6 x 150 mm column. The mobile phase was pumped at a rate of 1 ml.min⁻¹ and consisted of 550:370:80 acetonitrile:terahydrofuran:water. Sample volumes of 40 μ l were injected into the HPLC system and lycopene was measured as a single peak using an absorbance detector set at 460 nm. The run time was approximately 18 minutes. The retention time for lycopene was 9 min, considered to be all-*trans* lycopene, with lutein and zeaxanthin, cryptoxanthin and β -carotene at 2.2, 5 and 15 min respectively (Figure 6.3). Carotenoid

concentrations were calculated by using their respective standards (Hoffman-La Roche, Basel, Switzerland).

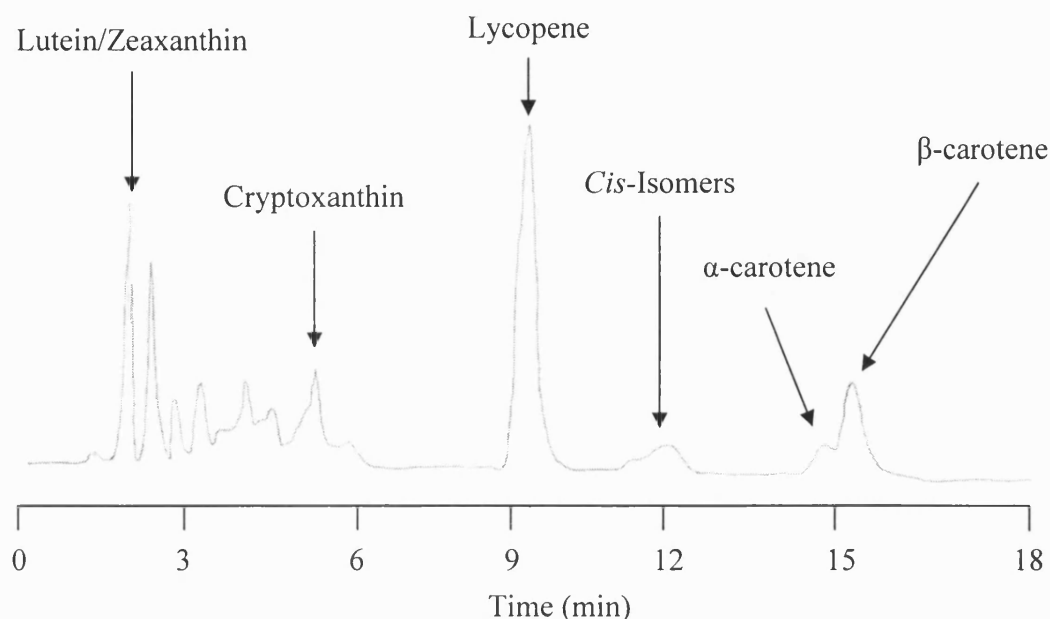


Figure 6.3 Chromatogram of carotenoid separation from human plasma samples.

6.2.5 Assessment of Physical Activity

The level of physical activity and total energy expenditure was assessed using a combined accelerometer and heart rate monitor (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK). This tool was worn for seven continuous days, collecting data measurements every minute during both day and night. The data was downloaded and analysed providing an estimate of daily energy expenditure and activity patterns (Actiheart 2.0, Cambridge Neurotechnology, Cambridge, UK). The software package produced two plots with data for basal metabolic rate (BMR) and active energy expenditure (AEE) (Figure 6.4). The data was transferred into an excel spreadsheet for further analysis. Basal metabolic rate (BMR) was calculated by the software using the Schofield equation (1985) with energy expenditure above sleeping heart rate (10th lowest point during sleep) contributing to AEE. This was calculated using branched equation models for heart rate and activity data, allowing total energy expenditure (TEE) to be determined.

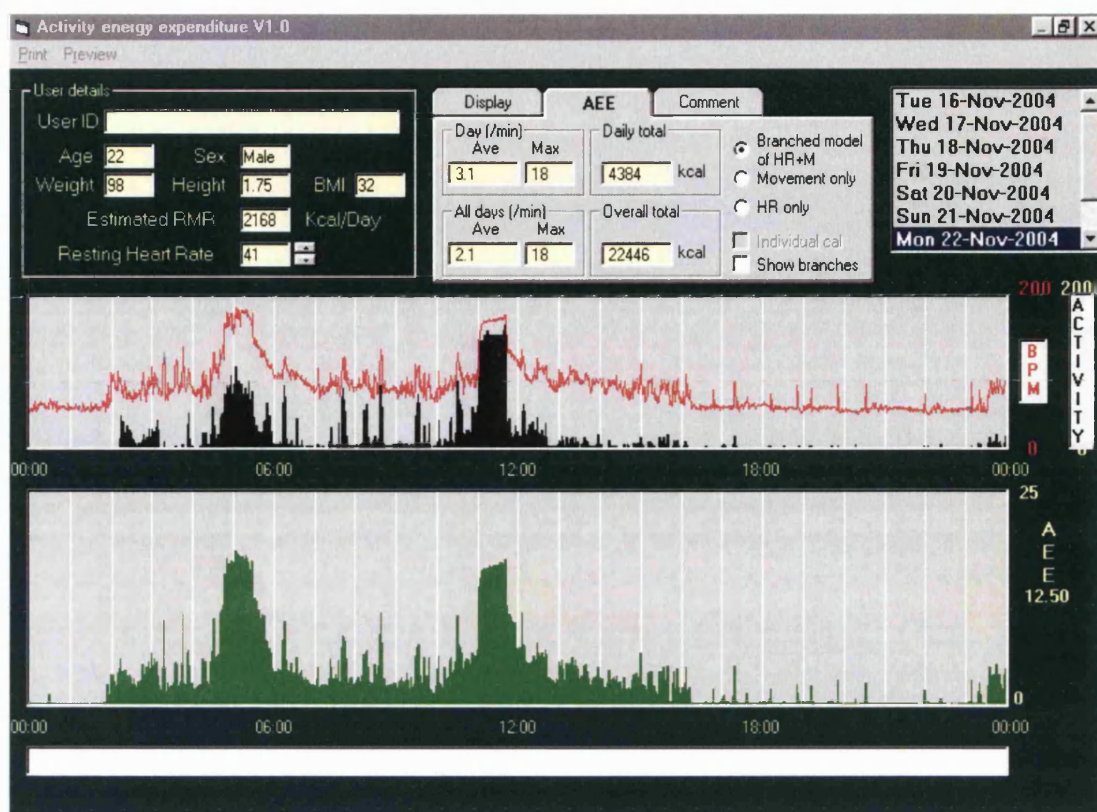


Figure 6.4 An example of the data analysis package used with the Actiheart monitors. The top figure is a plot of activity ($\text{kcal} \cdot \text{min}^{-1}$) and HR ($\text{beats} \cdot \text{min}^{-1}$) against time, with the shading representing activity and the line indicating HR. The bottom figure is a plot of AEE against time.

The combination of heart rate and accelerometry data in a branched equation model improves the estimate of physical activity energy expenditure compared with either method used alone or if the traditional non-branched combination is used (Brage *et al.*, 2004). In addition, branched modelling avoids individual calibration of these monitors (Brage *et al.*, 2004). The data output was provided as two separate plots of energy expenditure or heart rate against time and transferred to an excel file. A spreadsheet was used to calculate the TEE ($\text{AEE} + \text{BMR}$) as well as determine the number of min each subject spent above threshold values for 3, 6, 9, and 12 metabolic equivalents (METs) over the seven day testing period, providing daily and weekly estimates of TEE. Physical activity at an intensity less than 3, between 3 and 6, and greater than 6 METs reflected low, moderate and vigorous physical activity, respectively, as described by Pate and colleagues (1995). The data was analysed in $\text{kcal} \cdot \text{min}^{-1}$ and $\text{kcal} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$.

Physical activity recommendations recognise an accumulation of 10 min bouts of moderate activity to contribute to total daily activity, therefore only periods of 10 min or more were included in the duration at each intensity where HR was greater than 55% age-predicted maximum (Pollock *et al.*, 1998). Physical activity levels (PAL's) were calculated by dividing TEE by BMR providing another measure of physical activity. An example of the software analysis print out illustrates the differences in energy expenditure in subjects who are sedentary compared to very active (Figure A.5, Appendix 9).

6.2.6 Statistical Analysis

A repeated-measures ANOVA was used to compare results between the tomato-supplemented trial and no tomato trial, with a between-subjects factor of order of treatment. This was to ensure that there was no period effect from eating the tomato or no tomato diet first (Riso *et al.*, 1999). Effect size was calculated determining the magnitude of the change (Chapter 3.2.3).

Spearman's rank order correlation (ρ) was used to determine whether relationships existed between physical activity and levels of HO-1 protein expression and cell viability at baseline and following treatment. The relationship between plasma lycopene concentration and HO-1 protein expression and cell death were examined. The data were divided into the top 50% ($n=12$) and the lower 50% ($n=12$) based on activity duration (total activity time, moderate activity, and vigorous activity). A paired t statistic was calculated to examine whether there were differences between activity duration and basal levels of HO-1 protein and cell death, as well as the HO-1 and cell death response to treatment. In order to determine the effect of duration of activity (total activity, moderate activity only, or vigorous activity only) threshold values of 60 and 120 min per week were set and differences in the basal HO-1 protein and cell death at these limits were calculated using an unpaired t statistic.

Post-trial H_2O_2 -induced HO-1 was expressed as the fold change in MFI of the treated samples against the MFI of the sham-treated samples. Cell viability data was expressed as a percent. Values for HO-1 protein and cell viability following treatment were log-transformed as the data were not normally distributed. Significance was accepted at the 5% level. Values are presented as mean \pm S.D. ($n=24$). Data were analysed using SPSS version 12 (SPSS Inc., Chicago, USA).

6.3 Results

Plasma Carotenoid Concentration

Plasma carotenoid concentrations changed following supplementation with tomatoes (Figure 6.5). In the tomato trial, plasma lycopene and β -carotene concentrations increased significantly compared to the no tomato trials (ES = 1.38, $P = 0.000$ and ES = 0.17, $P = 0.004$ lycopene and β -carotene, respectively). Small differences in the levels of cryptoxanthin (ES = 0.28, $P = 0.034$) and canthaxanthin (ES = 0.36, $P = 0.021$) were also observed in the tomato condition.

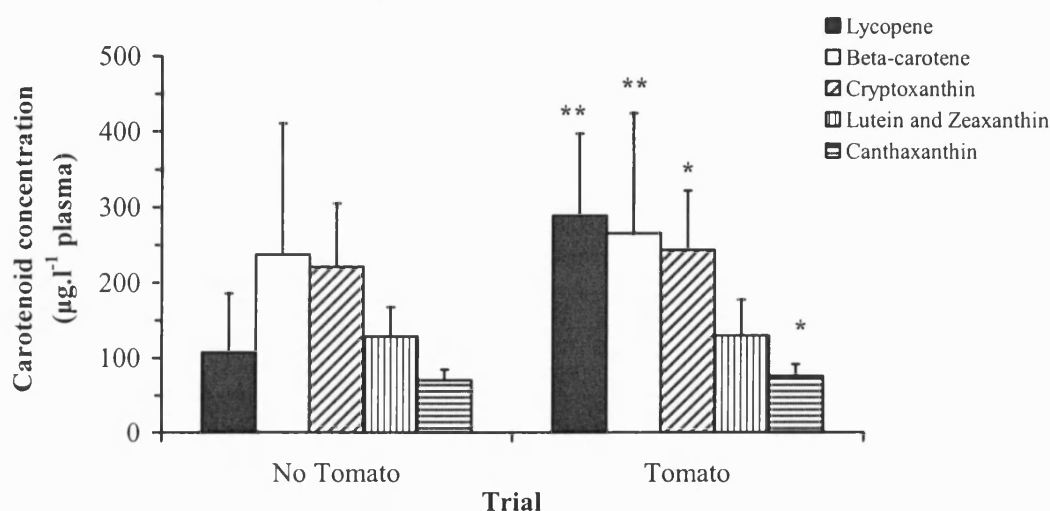


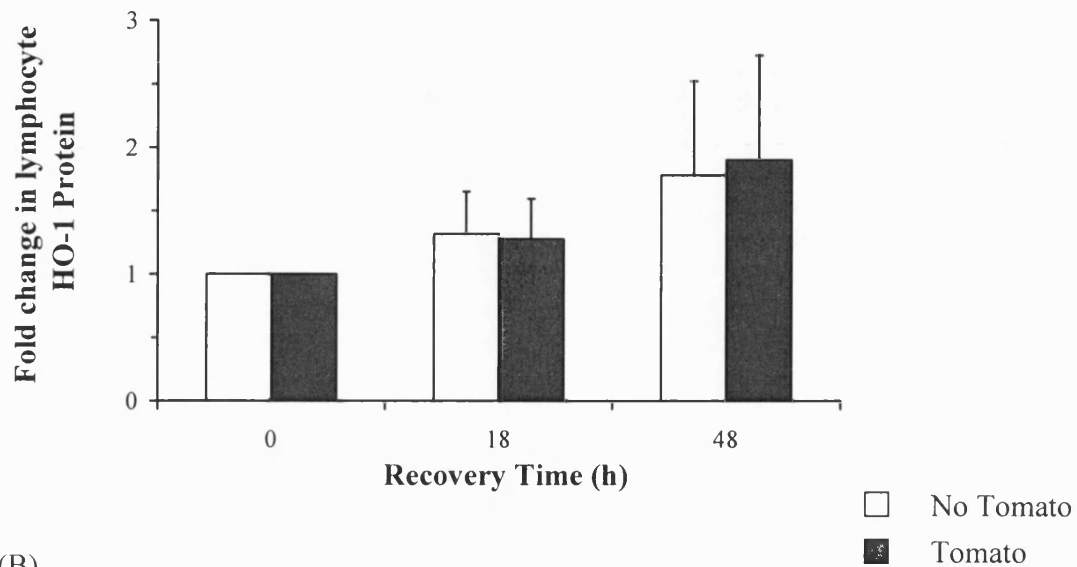
Figure 6.5 Plasma carotenoid concentration in the no tomato and tomato supplemented trials ($\mu\text{g.l}^{-1}$ plasma). ** denotes a significant difference in plasma concentrations between the tomato and control trials ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=24).

HO-1 protein expression

There was no difference in HO-1 protein expression in response to H_2O_2 treatment with 18 or 48 h recovery following the tomato supplemented or no tomato condition in both lymphocytes (ES = 0.12, $P = 0.713$ and ES = 0.16, $P = 0.881$ for 18 and 48 h respectively) and monocytes (ES = 0.02, $P = 0.715$ and ES = 0.20, $P = 0.572$ for 18 and 48 h respectively) (Figure 6.6). The level of HO-1 protein induction following 18 and 48 h recovery was significantly different to 0 h in both trials in lymphocytes ($P = 0.000$). In monocytes this was not significant following 18 h ($P = 0.929$ and $P = 0.729$ in the no tomato and tomato conditions, respectively) or 48 h recovery from

H₂O₂ treatment ($P = 0.099$ and $P = 0.062$ in the no tomato and tomato conditions, respectively).

(A)



(B)

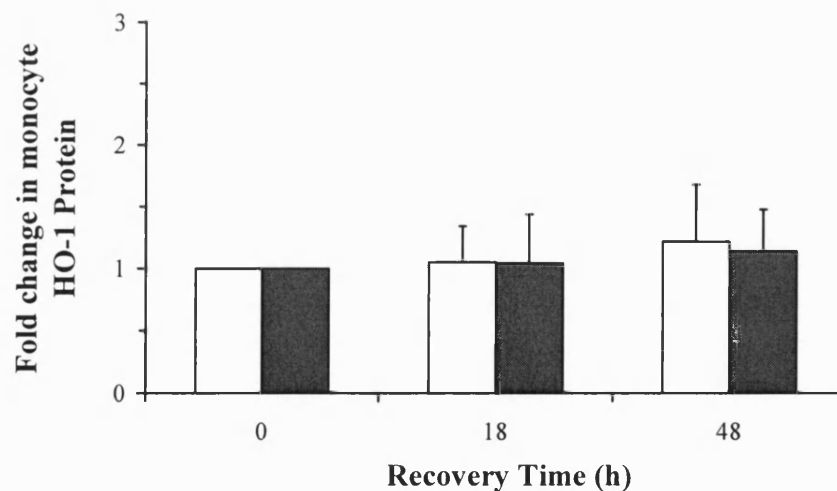


Figure 6.6 Fold change in lymphocyte (A) and monocyte (B) HO-1 protein expression at 18 h and 48 h following H₂O₂ treatment. Values are mean \pm S.D. (n=24).

There was no difference in the basal level of HO-1 protein in lymphocytes or monocytes in the tomato or no tomato condition (ES = 0.18, $P = 0.969$ and ES = 0.39, $P = 0.842$ for lymphocytes and monocytes, respectively) (Figure 6.7). However, a modest positive trend, assessed by Spearman's rank correlation (ρ), between plasma lycopene

concentration and basal lymphocyte HO-1 protein was evident in both the no tomato ($\rho = 0.250$, $P = 0.250$) and tomato-supplemented trials ($\rho = 0.356$, $P = 0.088$) (Figure 6.8).

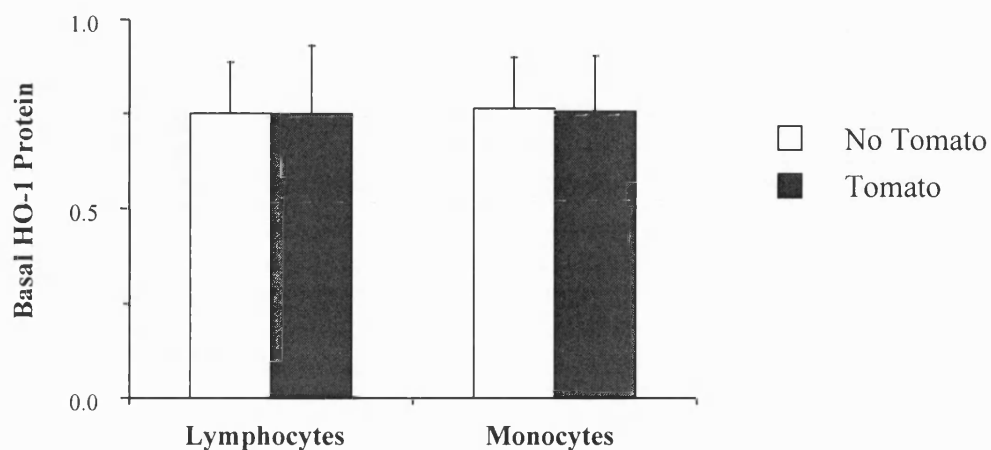


Figure 6.7 Basal level of HO-1 protein in lymphocytes and monocytes did not change following three weeks of tomato supplementation or no tomatoes. Values are mean \pm S.D. ($n=24$)

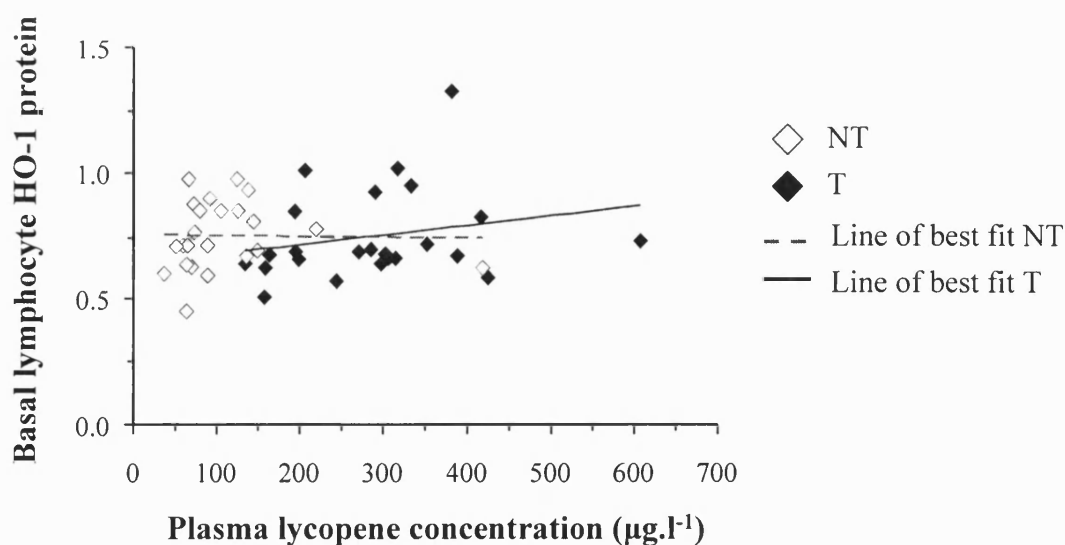
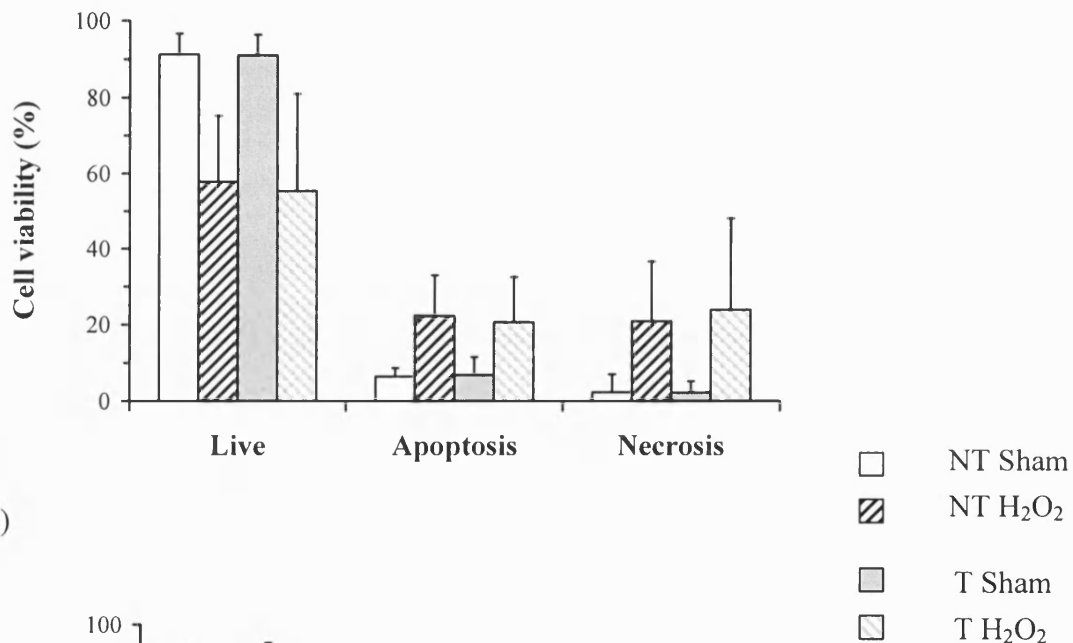


Figure 6.8 The relationship between plasma lycopene concentration and basal HO-1 protein expression in lymphocytes in the tomato supplemented ($\rho = 0.356$, $P = 0.088$) and no tomato trials ($\rho = 0.250$, $P = 0.250$). ($n=24$). NT: no tomato trial, T: tomato trial.

Lymphocyte apoptosis and necrosis

When lymphocytes were treated with H_2O_2 there was no difference in the percentage of live, apoptotic, and necrotic cells at 18 h (ES = 0.11, $P = 0.173$; ES = 0.15, $P = 0.275$ and ES = 0.13, $P = 0.353$ for live, apoptotic and necrotic cells, respectively) or 48 h recovery from treatment (ES = 0.04, $P = 0.876$; ES = 0.01, $P = 0.619$ and ES = 0.19, $P = 0.648$ for live, apoptotic and necrotic cells, respectively), following the tomato supplemented or no tomato conditions (Figure 6.9).

(A)



(B)

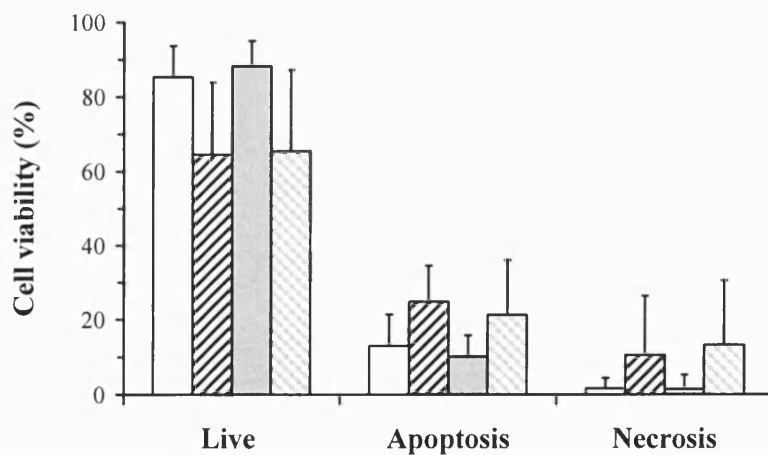


Figure 6.9 Percent live, apoptotic and necrotic cells in human lymphocytes at 18 h (A) and 48 h (B) following H_2O_2 treatment in tomato supplemented (T) and no tomato (NT) trials. Values are mean \pm S.D. ($n=24$ for 18 h, $n=19$ for 48 h).

A positive correlation was observed between plasma lycopene concentration and lymphocyte necrosis 18 h following H_2O_2 in both the tomato supplemented ($\rho = 0.438$, $P = 0.032$) and no tomato trials ($\rho = 0.444$, $P = 0.030$), respectively. In addition, a modest inverse relationship was found between plasma lycopene concentration and the proportion of live cells 18 h following H_2O_2 in both the tomato and no tomato trials ($\rho = -0.454$, $P = 0.026$ and $\rho = -0.496$, $P = 0.014$ for tomato and no tomato trials, respectively) (Figure 6.10). Figure 6.10 illustrates a shift to the right of the data points from the tomato trial with respect to the no tomato trial; therefore the tomato supplement did not have a protective effect on recovery from H_2O_2 treatment with no effect on the nature of the relationship. Similar trends were demonstrated following 48 h recovery from treatment in both the tomato ($\rho = -0.528$, $P = 0.020$, $\rho = 0.358$, $P = 0.132$ and $\rho = 0.451$, $P = 0.053$ for live, apoptotic and necrotic cells, respectively) and no tomato trials ($\rho = -0.307$, $P = 0.201$, $\rho = 0.293$, $P = 0.223$ and $\rho = 0.381$, $P = 0.108$ for live, apoptotic and necrotic cells, respectively).

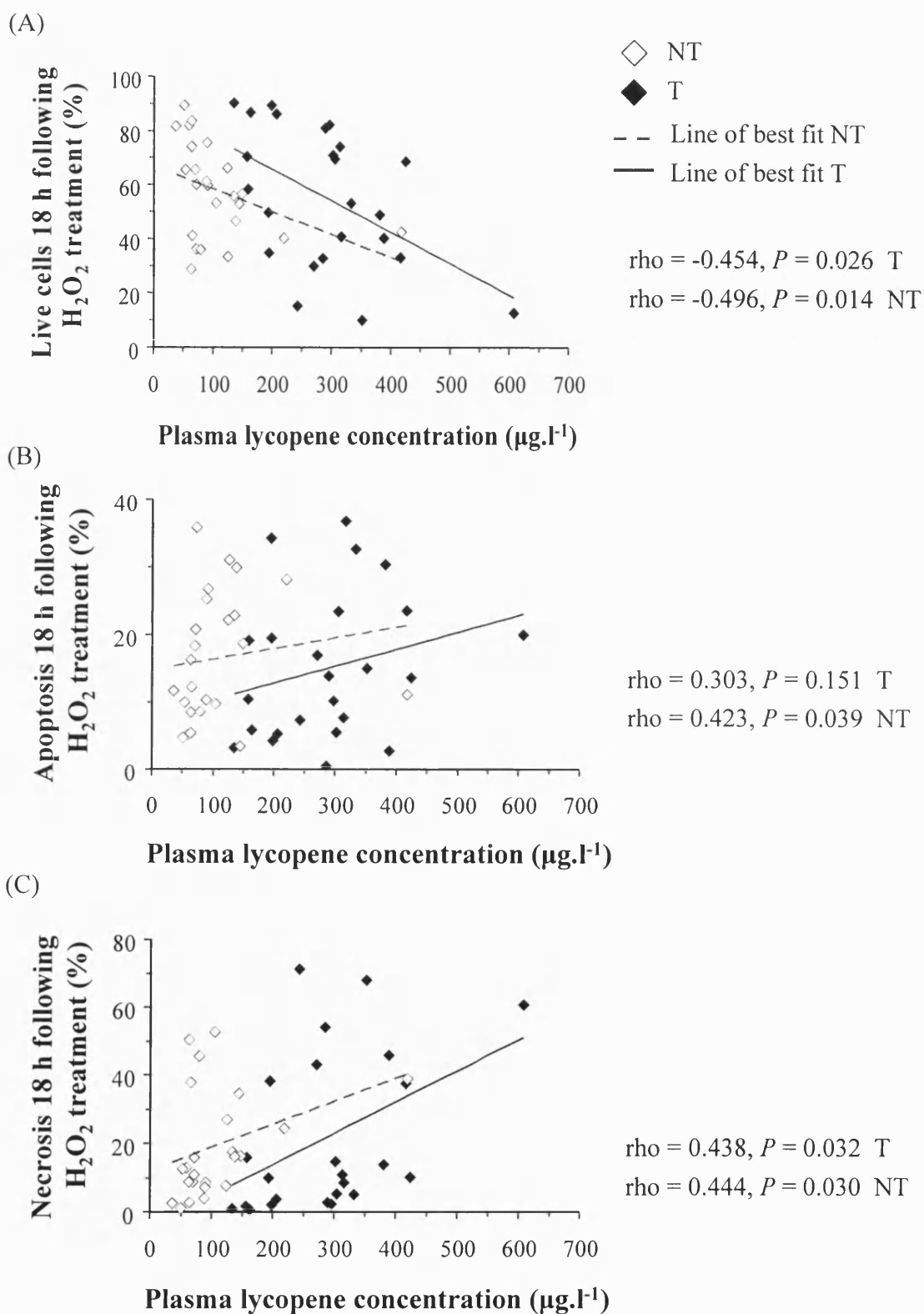


Figure 6.10 The relationship between plasma lycopene concentration and the proportion of live cells (A) apoptosis (B) and necrosis (C) 18 h following H_2O_2 treatment in the tomato supplemented (T) and no tomato trials (NT) ($n=24$).

In the untreated samples, the basal level of live cells increased with the tomato supplement (ES = 0.61, $P = 0.029$) and was accompanied by a reduction in the proportion of apoptotic cells (ES = 0.59, $P = 0.006$) (Figure 6.11). The proportion of necrotic cells in untreated lymphocytes in the tomato trial also reduced although this was not statistically significant (ES = 0.09, $P = 0.086$).

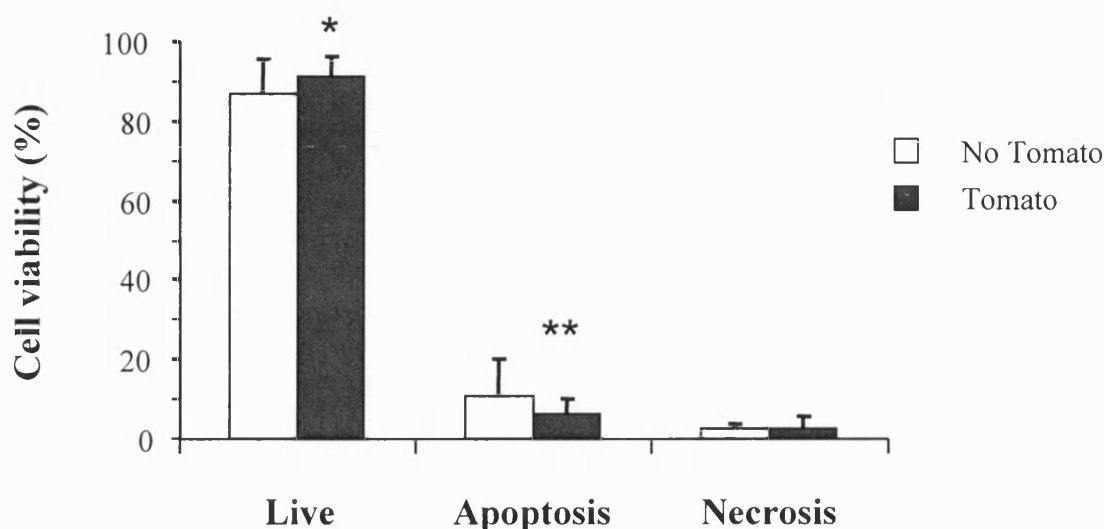


Figure 6.11 Basal level of live, apoptotic and necrotic lymphocytes following three weeks of tomato supplementation or no tomatoes. ** denotes a significant difference between the no tomato and tomato trial ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=24).

Physical activity and energy expenditure

Daily total energy expenditure (TEE) was 3909 ± 530 kcal (mean \pm S.D.) with estimated basal metabolic rate (BMR) ranging from $1535 \text{ kcal.day}^{-1}$ to $2168 \text{ kcal.day}^{-1}$ (Table 6.3). The duration of activity at moderate intensity or above (greater than three METS) was calculated for each person with the weekly duration of physical activity ranging from 10 min to 471 min (Figure 6.12).

Table 6.3 Daily total energy expenditure analysed as BMR, active energy expenditure (AEE) and physical activity level (PAL)

BMR (kcal.day ⁻¹)	AEE (kcal.day ⁻¹)	TEE (kcal.day ⁻¹)	PAL
1847 ± 168 (1535-2168)	2099 ± 425 (1393-2854)	3909 ± 530 (3019 to 5022)	2.1 ± 0.2 (1.6 to 2.6)

PAL represents the ratio between TEE and BMR. Values are mean ± S.D. (Range). (n=24).

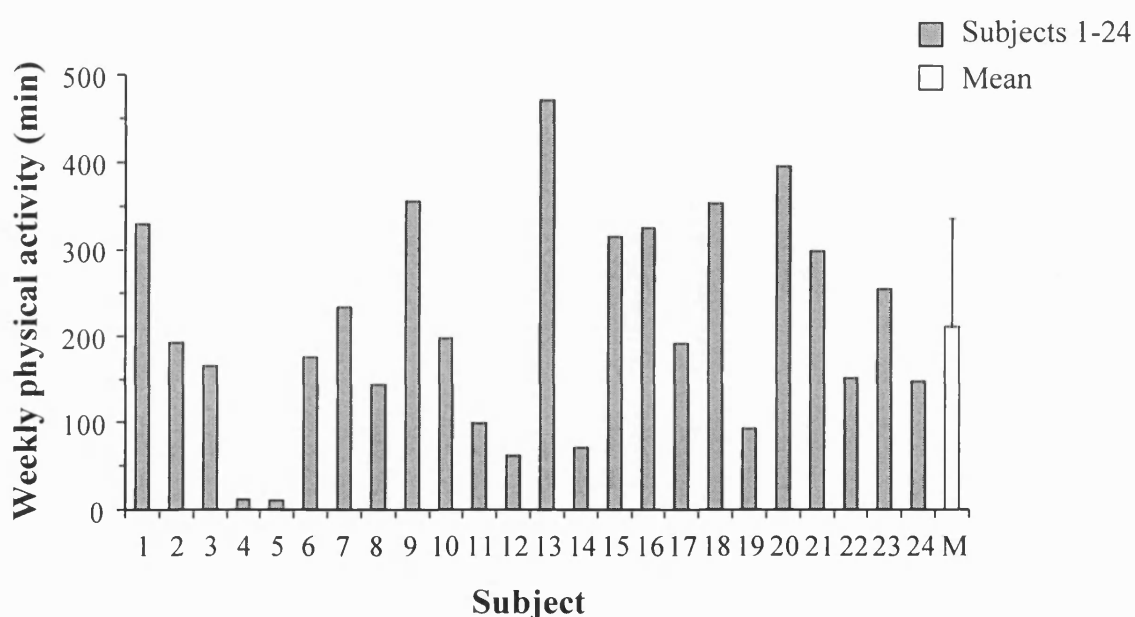


Figure 6.12 Weekly duration of physical activity at moderate intensity exercise or above, in subjects 1 to 24. M: mean ± S.D. Bouts of physical activity were only included when they exceeded 10 min at a HR greater than 55% age-predicted maximum in accordance with current ACSM exercise recommendations (Pollock *et al.*, 1998).

Physical activity recommendations of a minimum of 30 accumulated min of moderate intensity exercise at least five times a week is equivalent to 150 min of physical activity at three or more METS. The mean weekly exercise duration spent at or above moderate intensity was 210 ± 124 min (Table 6.4). Eight individuals failed to meet exercise recommendations with seven subjects participating in 300 min or more exercise a week.

Table 6.4 Duration of weekly activity (min) at differing intensity levels

Intensity	Moderate	Vigorous \longrightarrow		
METS	3-6	6-9	9-12	> 12
Time (min)	87 \pm 85	53 \pm 38	60 \pm 48	10 \pm 33

Values are mean \pm S.D. (n=24).

The proportion of each subject's time spent at different intensities was calculated with moderate intensity activity accounting for 43 \pm 29% of the weekly physical activity time (Figure 6.13). The amount of time spent participating in vigorous activities (intensity greater than six METS) accumulated to 57% of the total exercise duration.

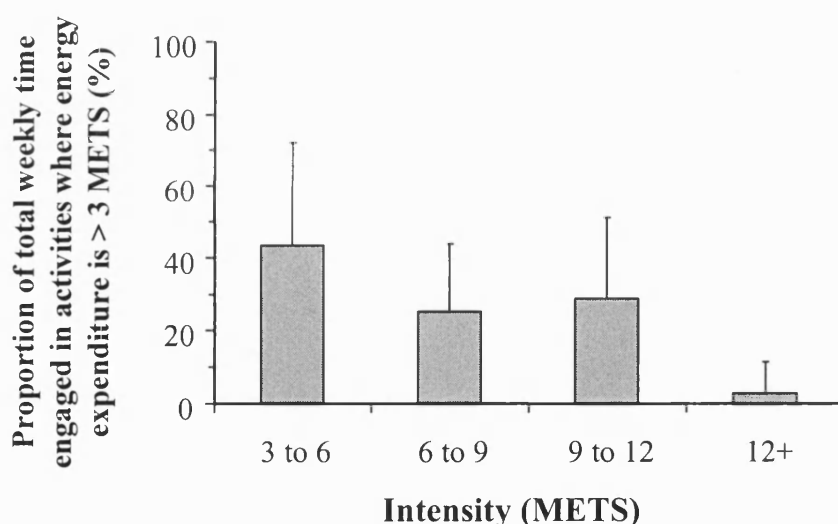


Figure 6.13 Proportion of weekly time engaged in moderate physical activity or above (> 3 METS). Values are mean \pm S.D. (n=24).

The data were analysed to determine whether there was a relationship between the weekly duration of exercise and basal levels of HO-1 protein, live, apoptotic and necrotic cells, and the responses to H₂O₂ treatment in human lymphocytes and monocytes. There was a positive relationship between the baseline basal measures of HO-1 protein and the time spent engaged in vigorous physical activity in both lymphocytes and monocytes. Additionally, it appears there may be a positive relationship between cell viability following H₂O₂ treatment and the amount of time

engaged in physical activity, however no other parameters measured showed consistent relationships across the different levels of physical activity (Table A.2, Appendix 10).

The baseline measure of basal HO-1 protein in lymphocytes and monocytes was modestly correlated with duration of time engaged in vigorous activity (intensity greater than six METS) ($\rho = 0.351$, $P = 0.093$ and $\rho = 0.413$, $P = 0.045$, in lymphocytes and monocytes, respectively) (Table A.2, Appendix 10). The sample was divided into two groups based on the amount of time spent engaged in vigorous activity (top and bottom 50%). The mean activity in the top 50% was 193 ± 59 min vigorous physical activity compared to 61 ± 39 min for the lower 50%. The basal level of HO-1 protein in lymphocytes and monocytes at baseline was also examined using threshold duration of vigorous physical activity set at arbitrary limits of 60 and 120 min (Figure 6.14). Significantly higher levels of basal HO-1 protein were found in lymphocytes and monocytes of individuals who engaged in more than 120 min vigorous activity ($P = 0.011$ and $P = 0.007$ for lymphocytes and monocytes, respectively) or were in the top 50% of activity durations ($P = 0.044$, and $P = 0.025$ for lymphocytes and monocytes, respectively).

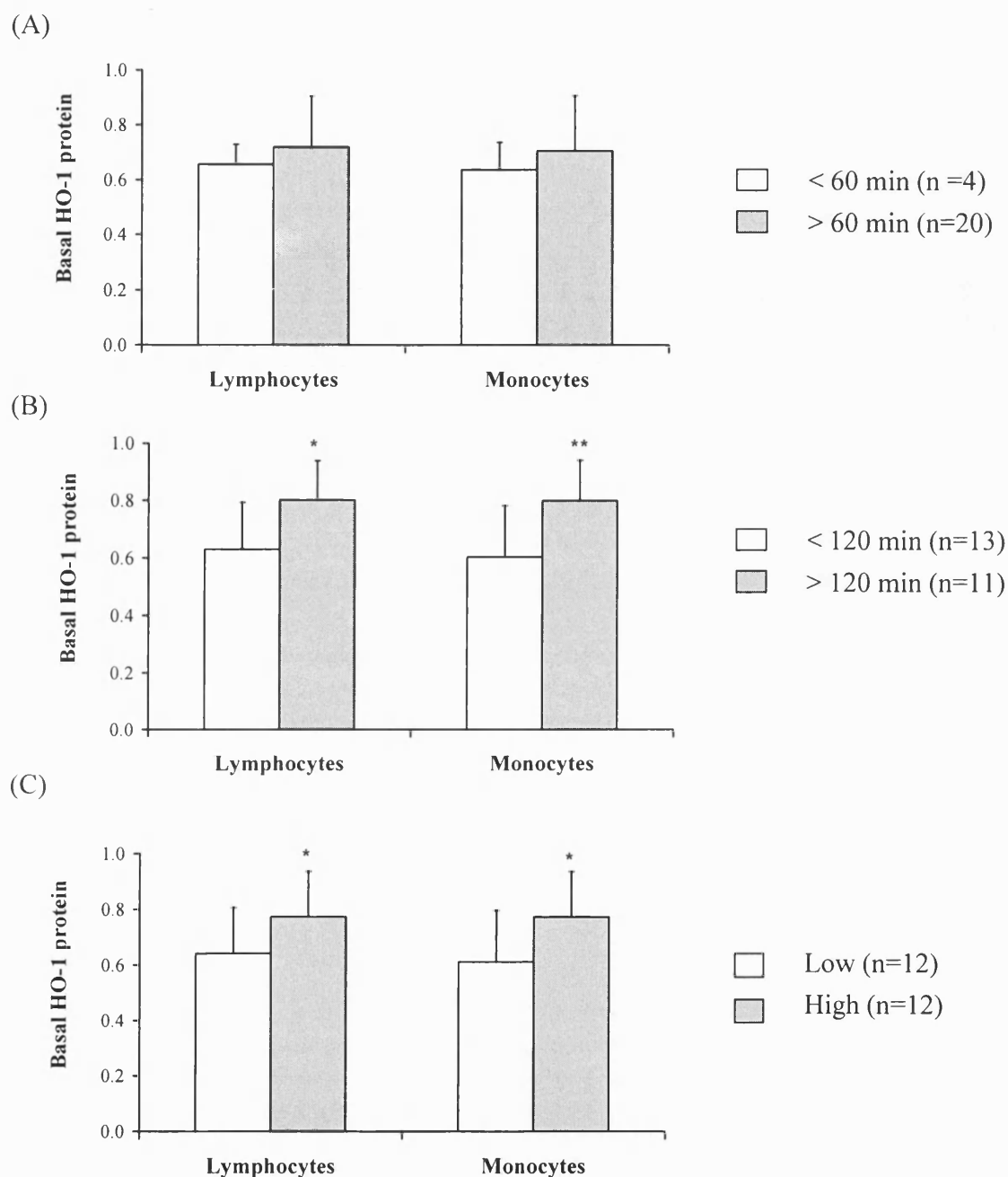


Figure 6.14 Threshold exercise duration of vigorous physical activity (intensity greater than six METS) associated with basal HO-1 protein in lymphocytes and monocytes. Thresholds of 60 min (A), and 120 min (B) were analysed. Groups relating to physical activity duration were formed with the top 12 durations in the high group (high) and the lowest 12 durations in the low group (low) (C). ** denotes a significant difference between basal HO-1 expression and threshold duration of vigorous exercise ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=24).

6.4 Discussion

In the present investigation, three weeks of intervention (tomato supplementation *versus* no tomatoes) resulted in an approximately three-fold higher level of plasma lycopene in the tomato supplementation condition demonstrating that plasma lycopene levels were successfully modulated. The HO-1 protein response to H₂O₂ treatment and the basal level of HO-1 protein were not affected by the intervention and, therefore, increased plasma lycopene concentration does not modulate HO-1 protein expression in lymphocytes and monocytes. Additionally, tomato supplementation did not alter cell viability following H₂O₂ treatment, although a significant reduction in apoptosis was found at basal levels in the tomato supplemented trial.

Epidemiological studies demonstrate an inverse relationship between tomato consumption and risk of cancer (Giovannucci, 1999; Lu *et al.*, 2001; Giovannucci *et al.*, 2002). Rao and Agarwal (1998) used a variety of tomato products providing up to 150 mg lycopene per day and demonstrated a reduction in lipid peroxidation (TBARS), protein thiols and DNA damage. Based on these findings, recommendations for a daily lycopene intake of 25-30 mg were made (Rao and Agarwal, 1999). Recently these observations have been demonstrated using low dose supplementation and an intake of 5 to 10 mg is recommended (Rao and Shen, 2002). In the present investigation, subjects were supplemented with 7 mg lycopene daily. This resulted in a 3-fold higher plasma lycopene concentration in the tomato trial. Other studies using between 5 and 30 mg lycopene daily have observed a 3-fold increase in plasma lycopene concentration (Gartner *et al.*, 1997; Chen *et al.*, 2001). Lycopene concentration of lymphocytes was not measured in the present investigation, although in a different study using the same amount of supplemented lycopene (and β -carotene), lymphocyte lycopene concentration increased ~2-fold (Porrini and Riso, 2000).

There is limited research examining changes in HO-1 expression following lycopene supplementation, although many studies have demonstrated that supplementation with other antioxidants has a suppressive effect on HO-1 induction (Ossola *et al.*, 1997; Pinot *et al.*, 1997; Yamaguchi *et al.*, 1997; Bornman *et al.*, 1999; Peng *et al.*, 2000). In the present investigation, tomato supplementation did not affect HO-1 protein induction in response to treatment with H₂O₂. As shown previously (Chapter 4), HO-1 protein induction increased significantly 48 h following recovery from treatment in both the tomato and no tomato trials. As the plasma lycopene concentration increased and did not

alter the HO-1 protein response to treatment, the findings from the present investigation show that HO-1 protein induction is not modulated by three weeks supplementation with tomatoes.

At present, only one human supplementation trial has reported a down-regulated expression of HO-1 protein in lymphocytes following oxidative stress (Peng *et al.*, 2000). In this investigation, subjects were supplemented daily with a mixed antioxidant, taken for five weeks. In the supplemented group, total plasma antioxidant status increased and was accompanied by reduced levels of MDA and protein carbonyl formation, reflecting an attenuation of oxidative stress. In the antioxidant supplemented group, exposure of lymphocytes to a free radical generator (AAPH) following initial heat shock, resulted in enhanced HSP synthesis and reduced HO-1 protein induction when compared to the responses of individuals prescribed the placebo. The findings from this study demonstrate that antioxidant status differentially modulates the synthesis of stress proteins. Khassaf and co-workers (2003) demonstrated lymphocytes from vitamin C supplemented individuals had a reduced induction of HSP60 and HSP70 synthesis in response to H₂O₂ treatment. This finding was supported by an increased expression of basal HSP60 and SOD and CAT activities in vitamin C supplemented subjects. According to the inducibility relationship presented by Boshoff and colleagues (2000), the increase in basal expression of HSP60 following supplementation may account for the decrease in its induction in response to H₂O₂ treatment. In the present investigation, tomato supplementation did not alter HO-1 protein induction following H₂O₂ treatment or the basal expression of HO-1 protein in lymphocytes or monocytes. The basal level of HO-1 protein was not reported in the study conducted by Peng *et al.* (2000) and so it is difficult to know whether the reduced induction of HO-1 that these authors observed with antioxidant supplementation was accompanied and/or explained by altered basal HO-1. Although previous research has shown that oxidant-responsive proteins can be altered through antioxidant supplementation, the findings from the present study did not observe any change in the level of HO-1 protein at baseline. Furthermore, this finding was supported by the lack of modulation of HO-1 protein induction following H₂O₂ treatment. Collectively, the current investigation demonstrates that three weeks of tomato supplementation does not modulate any aspect of HO-1 protein expression.

The findings reported from *in vitro* studies provide conflicting evidence regarding the effects of antioxidant supplementation and HO-1 expression. The addition of lycopene to the culture medium over a range of concentrations (0.1 to 8.8 μ M) led to a suppression of UVA-induced HO-1 mRNA at 100 and 250 kJ.m⁻² (Trekli, 2003). In

contrast, incubation with 0.5 μM lycopene led to an increased induction of HO-1 mRNA in skin fibroblasts following UVA irradiation (500 kJ.m^{-2}) (Offord *et al.*, 2002). As the doses of UVA used in these investigations were not the same, the discrepancy in the results may be explained by this factor. Interestingly, skin fibroblasts treated with epicatechin did not show differences in UVA-induced expression of HO-1 mRNA at intermediate doses although at high UVA doses (500 kJ.m^{-2}), epicatechin significantly increased HO-1 mRNA accumulation (Basu-Modak *et al.*, 2003). This increase in HO-1 mRNA may reflect an increase in oxidative stress with antioxidant treatment. It is possible that antioxidants provide protection against modest oxidative stress, whereas during more severe oxidative stress antioxidants may interact with oxidants to exacerbate oxidative stress. According to this contention, antioxidant treatments with stimuli that induce a high degree of oxidative stress may not be protective and could lead to augmented oxidative stress. Within this context it is noteworthy that in the present investigation, a non-lethal dose of H_2O_2 was used to induce HO-1 and this was unaffected by tomato supplementation.

Apoptosis is induced in human lymphocytes following *ex vivo* treatment with H_2O_2 (Marini *et al.*, 1996a). In the present investigation, apoptosis was induced in human lymphocytes 18 h following recovery from *ex vivo* treatment with H_2O_2 . Lycopene supplementation provides protection against H_2O_2 -induced DNA damage (Riso *et al.*, 1999; Porrini and Riso, 2000) and therefore may modulate cell viability following H_2O_2 treatment. In the present study, tomato supplementation did not affect the proportion of live, apoptotic and necrotic lymphocytes in response to treatment with H_2O_2 . Although plasma lycopene concentrations increased, the degree of oxidative stress induced by H_2O_2 treatment was the same in both trials as HO-1 protein was induced to the same level. Therefore, the present findings demonstrate that tomato supplementation does not modulate the mode of cell death in response to H_2O_2 treatment and that this may represent the failure of lycopene to successfully modulate the degree of oxidative stress induced by hydrogen peroxide treatment.

Interestingly, in basal conditions, there was a significant increase in the proportion of live lymphocytes and a decrease in the percentage of apoptotic lymphocytes after three weeks of tomato supplementation. It is not clear what explains these results, although, because HO-1 remained unchanged, it seems unlikely that this was through an oxidant-mediated mechanism. Many hormones and growth factors are capable of inducing and facilitating apoptosis under physiological or pathological conditions, or both (Kieiss and Gallaher, 1998). The protection afforded by lycopene may involve hormonal and immune system modulation (Rao and Agarwal, 1999). Previous research has

demonstrated lycopene interferes with autocrine/paracrine loop by down-regulating sex steroid hormones and growth factor activation and signalling in the prostate (Siler *et al.*, 2004). Hence, in the present study, it is possible that reductions in the level of circulating hormones such as insulin-like growth factor-1 (IGF-1) may be the cause of the decrease in apoptosis at basal levels. Apoptosis is essential to normal development and removes dysfunctional cells. A reduction in apoptosis may not necessarily be a beneficial process; however, current therapies shown to improve patient outcomes in atherosclerosis reduce apoptosis in atherosclerosis as part of their action (Stoneman and Bennett, 2004). As lymphocytes play an important role in early atherosclerosis it is possible that lowered lymphocyte apoptosis is beneficial. However, based on the findings reported in this thesis, it is not possible to either explain the reduction in apoptosis or draw conclusions about the significance of this finding.

In the present investigation, the level of physical activity undertaken by each subject was estimated over a one week period using a combined heart rate monitor and accelerometer. People who are regularly active obtain beneficial adaptations from exercising which protect them against the risk of disease. These adaptations include an increase in the level of endogenous antioxidant enzymes, lowering the pro-oxidant state of the body (Powers *et al.*, 1999; Powers and Sen, 2000; Miyazaki *et al.*, 2001). Therefore, individuals who take part in regular exercise may respond to oxidative stress in a different manner to those individuals who are inactive. Furthermore, if individuals have gained adaptations to exercise, they may respond differently to antioxidant supplementation. In the present investigation, the duration of weekly physical activity ranged from 10 min of moderate intensity activity to 471 min of activity consisting of moderate and high-intensity work. Current exercise recommendations suggest a minimum of 30 min accumulated moderate intensity activity at least five times a week (Brooks *et al.*, 2004; DoH, 2004). In the current study, eight people did not fulfil the recommended criteria, participating in less than 150 min of moderate intensity exercise per week.

Previous research has shown that highly trained athletes have a lower basal level of HO-1 protein (Niess *et al.*, 1999b) and a higher level of apoptosis at rest (Mooren *et al.*, 2004). In the present study, individuals that engaged in more than 120 min of vigorous physical activity per week had significantly higher levels of basal HO-1 protein in both lymphocytes and monocytes. Interestingly, five consecutive days of one-legged knee extensor exercise increased basal HO-1 mRNA in skeletal muscle by 2-fold when compared to the control leg (Pilegaard *et al.*, 2000). This finding demonstrates that the exercised leg was exposed to oxidative stress resulting in an increase in HO-1.

Consequently, this may represent an adaptation to regular exercise or be related to the last acute bout of exercise. Fehrenbach and colleagues (2003a) demonstrated that the basal level of HO-1 protein was different in two groups of endurance exercisers. In a previous investigation, the same group showed that basal HO-1 protein was lower in trained athletes prior to participating in a half marathon (Niess *et al.*, 1999b). In contrast, the second paper by this group showed that trained endurance athletes in the eccentric exercise group did not show lower basal HO-1 protein when compared to the untrained group (Fehrenbach *et al.*, 2003a). Interestingly, the untrained group participated in less than 3 h physical activity per week (Fehrenbach *et al.*, 2003a) and, in the present investigation, it was demonstrated that 2 h vigorous physical activity per week was sufficient to increase basal HO-1 protein. Additionally, the amount of time in which subjects refrained from exercise differed in these studies, ranging from three days in the present investigation up to one week (Fehrenbach *et al.*, 2003a). Ultimately, this could affect basal HO-1 protein as the length of time required for the acute responses to exercise to return to 'normal' conditions is not known and this needs to be investigated in order to be able to effectively compare the basal level of HO-1 protein in groups of active individuals.

The present work did not find any other relationships between physical activity and cell viability or the responses to H₂O₂ treatment. However, in the current study the subjects recruited were mostly active and it may be possible that tomato supplementation of physically active people does not affect their responses *versus* no supplementation, as these individuals already benefit from increased antioxidant protection afforded through regular exercise. It is plausible that antioxidant supplementation of habitually active people is ineffective because of adaptations (Clarkson and Thompson, 2000). Alternatively, antioxidant supplementation could also simply replace endogenous defences and this would probably not be justified. However, sedentary people do not benefit from these adaptations and may benefit from supplementation if they are not convinced to become more active. Therefore, future work should look at supplementation in sedentary populations who have not benefited from the adaptations to regular exercise.

In summary, tomato supplementation increases plasma lycopene concentration although this does not modulate the level of basal or induced HO-1 protein in human lymphocytes and monocytes. In addition, the mode of cell death in response to H₂O₂ treatment was unaffected by tomato supplementation. However, basal levels of lymphocyte apoptosis were significantly reduced in the tomato supplemented trial. This finding appears to be mediated by a non-oxidative mechanism of lycopene since the expression of the

oxidant-sensitive protein HO-1 was unchanged. Individuals engaging in more than 120 min of vigorous physical activity had higher basal levels of HO-1 expression. As the level of physical activity of the subjects used in this trial was high, this may have affected the response to tomato supplementation. The WHO (2002) estimate that approximately 60% of the global population do not achieve current physical activity guidelines. This large inactive population are more at risk of developing chronic diseases and therefore, future investigations should examine whether antioxidant supplementation is effective in modulating the expression of oxidant-responsive genes in this group of people. Furthermore, because of the effect of regular exercise on antioxidant defences, it is recommended that researchers should not look at antioxidant supplementation in isolation without considering the effects of regular physical activity.

CHAPTER 7

GENERAL DISCUSSION

A common element of all inducers of haem oxygenase-1 (HO-1) is their ability to induce oxidative stress (Morse and Choi, 2002). Consequently, HO-1 expression is a marker of oxidative stress (Tyrrell and Basu-Modak, 1994; Offord *et al.*, 2000). Conditions which disturb the balance between free radicals and antioxidants will result in oxidative stress and, therefore, affect the expression of HO-1. In this thesis, two models of oxidative stress were employed in human mononuclear cells; (i) a 'whole body' approach using acute exercise and (ii) *ex vivo* H₂O₂ treatment. The *ex vivo* model was superimposed upon the exercise model to determine whether an acute bout of demanding exercise alters the response to an exogenous oxidant challenge. The *ex vivo* treatment model was judged to be a better inducer of HO-1 protein when compared to acute exercise and therefore this model was subsequently used with tomato supplementation to investigate whether it was possible to modulate the induction of HO-1.

The main findings reported in each Chapter of this thesis are listed below:

- HO-1 mRNA induction in mononuclear cells is variable following a half marathon race (Chapter 3).
- The basal level of HO-1 mRNA is higher prior to exercise than rest (Chapter 3).
- The induction of HO-1 protein in human lymphocytes *ex vivo* is maximal 48 h following exogenous H₂O₂ treatment and this is a reproducible response (Chapter 4).
- The induction of HO-1 mRNA following intermittent exercise is variable and was not accompanied by an increase in HO-1 protein (Chapter 5).
- At 2 h post-exercise, there is a reduced ability of lymphocytes to induce HO-1 protein induction 48 h following *ex vivo* H₂O₂ treatment (Chapter 5).
- Tomato supplementation increased plasma lycopene concentration and did not alter any aspect of HO-1 protein expression (Chapter 6).
- In basal conditions, the proportion of live lymphocytes increased and the percentage of apoptotic lymphocytes decreased following tomato supplementation (Chapter 6).
- Individuals who engaged in vigorous physical activity levels had higher basal HO-1 protein levels at baseline (Chapter 6).

The exercise-induced increase in HO-1 mRNA in human mononuclear cells appears to be a variable response (Chapters 3 and 5). This observation was also demonstrated by Thompson and colleagues (2005) following continuous running at 70% $\dot{V}O_{2\max}$ where one subject had a pronounced response of a 20-fold increase in HO-1 mRNA 24 h post-exercise. There are several possible explanations why individuals respond differently to exercise. The subjects used in the current investigations had either been training specifically for a half marathon race (Chapter 3) or habitually participated in intermittent activities in their leisure time (Chapter 5). Inevitably, the absolute amount of training and the quality of training varied between individuals and it is possible that the varied HO-1 response to an acute bout of exercise is partly explained by differences in training. Regular exercise leads to adaptations including increased levels of antioxidant enzymes (Powers and Sen, 2000) and antioxidant molecules such as vitamin C (Robertson *et al.*, 1991) and this may lead to differences in the degree of oxidative stress induced by an acute bout of exercise. Alternatively, there may be a genetic explanation for these differences. Recently, a polymorphism in the promoter region of the HO-1 gene has been discovered (Yamada *et al.*, 2000; Kaneda *et al.*, 2002; Hirai *et al.*, 2003; Exner *et al.*, 2004) and this has been shown to influence the inducibility of the HO-1 response.

Interestingly, the results presented in this thesis further confirm the variability in HO-1 induction since, although HO-1 induction 48 h following H_2O_2 treatment was a reproducible response, there was considerable inter-individual variability with the fold increase in HO-1 protein in lymphocytes ranging from 1.4 fold to 4.6-fold (Chapter 4). Inter-individual variability in lymphocyte HO-1 protein induction has been shown in several investigations using H_2O_2 (Marini *et al.*, 1996a), heavy metals (Menzel *et al.*, 1998) and HBO (Speit *et al.*, 2000). This variability in HO-1 induction may be a useful property in that this clearly shows that people respond to oxidative stress in differing degrees. However, it is not known to what degree variations in the ability to induce HO-1 are related to differences in the degree of oxidative stress experienced by tissues or other factors such as genotype. It is possible that the variability in HO-1 induction may affect the use of this gene as a marker of oxidative stress in cross-sectional studies where it may be used as a stand alone measurement taken on a single occasion. Consequently, a measure of HO-1 induction in isolation may be meaningless and this should be investigated further to strengthen the application of HO-1 in these types of studies. However, HO-1 provides a useful marker of oxidative stress in trials of a cross-over design such as those employed in the present thesis where people act as their own controls.

Acute exercise initiates oxidative stress and an inflammatory response. In principle, it is possible that the increase in HO-1 mRNA may be directly mediated by exercise-induced oxidative stress during the exercise itself or that HO-1 induction is a response to oxidative stress caused by exercise-induced inflammation. Previous investigations have shown prolonged exercise induces HO-1 mRNA (Thompson *et al.*, 2005) and HO-1 protein (Niess *et al.*, 1999b; Thompson *et al.*, 2005) in human lymphocytes. Both these studies demonstrated an increase in pro-inflammatory cytokines, with one of these investigations reporting a positive relationship between plasma IL-8 concentration and HO-1 protein induction (Niess *et al.*, 1999b). In the present thesis, both acute exercise protocols demonstrated significant increases in plasma IL-6 concentration post-exercise (Chapters 3 and 5). Interestingly, a positive relationship was observed between the fold increase in plasma IL-6 and the peak fold increase in HO-1 mRNA accumulation following a half marathon race and intermittent running. When the results for both trials are pooled, a modest positive relationship was found between the fold change in peak HO-1 mRNA and fold change in peak plasma IL-6 ($r = 0.536$, $P = 0.032$) (Figure 7.1).

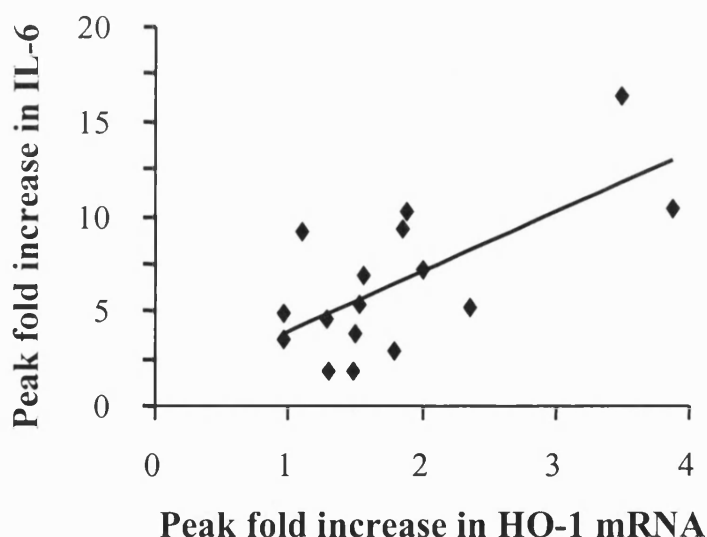


Figure 7.1 The relationship between the peak fold increase in IL-6 concentration and the peak fold increase in HO-1 mRNA expression following acute exercise ($r = 0.536$, $P = 0.032$) ($n=16$, pooled data from Chapter 3 and Chapter 5).

Although a positive relationship between the anti-inflammatory cytokine IL-10 and HO-1 mRNA expression was demonstrated, this was not statistically significant (Chapter 5). Collectively, these results tentatively suggest that induction of HO-1 in lymphocytes following exercise may be related to the pro-inflammatory effect of an acute bout of exercise. With this in mind, it is also possible that the delayed induction of HO-1 protein in the *ex vivo* model could be mediated by cytokine-induced oxidative

stress. This delayed induction was demonstrated in osteoarthritic chondrocytes following incubation with IL-10 (Fernandez *et al.*, 2003) and therefore, HO-1 induction may mediate the protective effects of IL-10. Within this context, and because it appears that HO-1 is directly responsible for the anti-inflammatory effects of IL-10 (Lee and Chau, 2002), future work should investigate the relationship between pro- and anti-inflammatory cytokines and the induction of HO-1 *in vivo* and *in vitro*.

Acute exercise induces an immune response which is characterised by changes in the cell number of leukocyte subpopulations (Field *et al.*, 1991; Hoffman-Goetz and Pedersen, 1994). In the hours following exercise, there is a decline in the number of lymphocytes which generally falls below normal levels. Several investigations have shown that lymphocyte responses to mitogen stimulation are lowered in the recovery period (0-2 h) following exercise (Field *et al.*, 1991; MacNeil *et al.*, 1991; Nielsen, 2003). The post-exercise decline in lymphocyte concentration is not accompanied by a compensatory increase in lymphocyte function and, consequently, the lymphocyte function in the blood is considered to be suppressed (Pedersen and Hoffman-Goetz, 2000). This suppression of immunity post-exercise may last between 3 and 72 h and is often described as an 'open window' to infection (Nieman and Pedersen, 1999). In the present thesis, 2 h post-exercise the induction of HO-1 protein following *ex vivo* H₂O₂ treatment was significantly reduced when compared to the corresponding time point in the rest trial (Chapter 5). The 2 h time point coincided with the time at which lymphocyte cell number was lowest. Although speculative, one possible interpretation of this phenomenon is that the lack of induction of HO-1 is in some way directly involved in post-exercise immuno-suppression. The failure to induce HO-1 could be representative of a general suppression of cellular responses to any given stimulus and indicate a maladaptive response to demanding exercise.

Alternatively, the lower induction of HO-1 protein following *ex vivo* treatment post-exercise may be related to other protective mechanisms which are induced by demanding exercise. For example, acute exercise increases the expression of antioxidant enzymes in lymphocytes (Tauler *et al.*, 2003) and has been shown to up-regulate the expression of HSP in leukocytes immediately following prolonged running (Fehrenbach *et al.*, 2000a; Fehrenbach *et al.*, 2000b). Both these responses enhance the antioxidant defence system and this increased protection may attenuate the degree of oxidative stress induced by a subsequent stressor, resulting in a lower induction of HO-1 to a second stimulus. Therefore, it is possible that exercise 'conditions' lymphocytes and generates an adaptive response that protects lymphocytes against subsequent oxidative stress. As the increase in HO-1 mRNA following intermittent exercise was not as large

as anticipated and was not accompanied by increased HO-1 protein, it is possible that the reduced ability of lymphocytes to induce HO-1 protein in response to *ex vivo* treatment would be greater if prolonged exercise was used as the initial HO-1 inducing stimulus. Additionally, a more pronounced decline in lymphocyte concentration would occur and this may represent an increased susceptibility to subsequent oxidative challenge *in vivo*.

In the present thesis, the induction of HO-1 in response to treatment was not related to the basal expression of the gene (Chapters 3 and 5). In fact, the basal level of HO-1 mRNA accumulation was significantly higher prior to the half marathon race and this may have masked the induction of HO-1 mRNA post-exercise (Chapter 3). It is possible that the higher basal HO-1 mRNA in these individuals can be explained by the intense period of training in the months preceding the race and that one of the adaptations to regular exercise is an increase in basal HO-1. This adaptive response to regular exercise was demonstrated in skeletal muscle with a 2-fold increase in HO-1 mRNA found in the exercised leg when compared to the control leg following five days of consecutive exercise (Pilegaard *et al.*, 2000). Alternatively, it is possible that increased basal expression of HO-1 mRNA is influenced by anxiety prior to competition in the knowledge that demanding exercise is imminent. Since catecholamines are known to be powerful pro-oxidants (Halliwell and Gutteridge, 1999), it is possible that they induce HO-1. Although there is no evidence that catecholamines induce HO-1 in leukocytes, several investigations have demonstrated that catecholamines induce HO-1 mRNA accumulation and HO-1 protein in endothelial cells and astrocytes *in vitro* (Schmidt *et al.*, 1999; Berger *et al.*, 2000; Khorchid *et al.*, 2002). Indeed, increased basal HO-1 was observed in seven out of eight individuals prior to the exercise trial (Chapter 5). As trial order was randomised and had no effect on basal HO-1 expression, this finding was related to the exercise trial alone. One implication from this finding is that in future work subjects should arrive in the laboratory without knowing whether they will participate in rest or exercise trials.

An increased level of basal HO-1 protein was demonstrated in individuals who engaged in vigorous physical activity (> 6 METS) (Chapter 6). One potential explanation for this phenomenon is that regular vigorous physical activity increases the expression of HO-1 through repeated 'exposure' to exercise-induced oxidative stress and that the increase in HO-1 is similar to the increases previously observed for antioxidant enzymes such as SOD (Miyazaki *et al.*, 2001). In contrast, other investigators have reported a lower level of HO-1 protein in leukocytes of trained athletes (Niess *et al.*, 1999b; Fehrenbach *et al.*, 2003a) and this was suggested to represent the lower pro-oxidant state of the athlete.

Ostensibly, it may appear difficult to reconcile these contradictory findings. However, it is possible that the expression of oxidant-responsive genes in highly trained athletes is different to individuals who are 'regularly active' (Chapter 6). With this in mind, Sen (1995) suggested that elite athletes may benefit from antioxidant supplementation as the changes derived from regular exercise training may not be enough to suppress exercise-induced oxidative stress. Alternatively, the differences in basal HO-1 protein may be related to the effects of the last acute bout of exercise, as the time for these responses to return to baseline levels is not known. In the studies showing that trained individuals had lower basal levels of HO-1 protein, subjects refrained from 'intense' exercise for up to one week, whereas in the present investigation this was three days. Because of the potential importance of HO-1 in lymphocyte and monocyte function, future experiments should seek to determine whether there is a dose-response between exercise and HO-1 expression, and whether extremely demanding regular exercise is associated with suppressed basal HO-1.

Antioxidant supplementation enhances the body's natural defence system and can attenuate oxidative stress. Traditionally, it is believed that excessive free radical production is 'harmful' and that antioxidants are beneficial to cells (Jackson *et al.*, 2002). The regulation of oxidant-responsive genes is determined by redox status and therefore, antioxidant supplementation has the potential to modulate the expression of these genes (Jackson *et al.*, 1998; Jackson *et al.*, 2002). As genes such as HO-1 are up-regulated and offer protection to cells, it is not known whether altering the response to oxidative stress through antioxidant supplementation is beneficial or deleterious to the tissue (Khassaf *et al.*, 2003). In the present thesis, tomato supplementation significantly increased plasma lycopene concentration but did not modulate HO-1 expression at basal levels or in response to H₂O₂ treatment (Chapter 6). The subjects used in the present investigation were mostly active; two thirds of individuals attained exercise recommendations with ~50% of this group achieving double the amount of 'recommended' minutes at the appropriate intensity per week. As the HO-1 protein response in these individuals did not differ with or without tomato supplementation it is possible that individuals who are vigorously active and have benefited from adaptations to regular exercise do not benefit further from supplementation. There is evidence that people who are physically active are protected against many of the same diseases where lycopene also offers protection (Erikssen *et al.*, 1998; Hu *et al.*, 2000; Wannamethee *et al.*, 2001; Lee, 2003) and therefore, it is possible that people who are active 'regularly' already have increased antioxidant protection. It is not known whether people who participate in mostly moderate physical activity respond to antioxidant supplementation and this is an area which warrants further investigation. Perhaps this could be examined

by adopting a differentiated supplementation study whereby antioxidants are provided to people who choose to be sedentary or are highly active.

Physical inactivity potentiates at least 20 unhealthy conditions (Booth *et al.*, 2002) and, since oxidative stress is also involved in many of these conditions, people leading a sedentary lifestyle may benefit from antioxidant supplementation. In the present thesis, tomato supplementation significantly lowered basal lymphocyte apoptosis without modulating the expression of HO-1. Therefore, it appears that the reduction in apoptosis occurred via a non-oxidative mechanism derived from lycopene supplementation. Apoptosis is induced by a number of hormones (Kiess and Gallaher, 1998) and therefore it is possible that the reduction in apoptosis may be related to decreased levels of circulating hormones, such as IGF-1 and prostate specific antigen (PSA), observed following lycopene supplementation (Bowen *et al.*, 2002; Siler *et al.*, 2004; Tang *et al.*, 2005). Apoptosis is a functionally important mechanism designed to eliminate non-functional or abnormal cells and, ostensibly, this may not seem a beneficial effect of tomato supplementation. However, with reference to the cancer literature, the therapeutic gain achieved by suppressing apoptosis in normal tissues may be just as important as that gained through increasing apoptosis in cancerous tissues (Milas *et al.*, 1994). Furthermore, lymphocytes play an important role in atherosclerosis (Song *et al.*, 2001) and anti-apoptotic therapy may reduce the initiation, progression or clinical consequences of atherosclerosis (Stoneman and Bennett, 2004). Although it is premature to draw major conclusions from the present investigation, it is noteworthy that high lycopene intakes are associated with reduced risk of cardiovascular disease (Kohlmeier *et al.*, 1997; Rissanen *et al.*, 2003). Therefore, in some instances, a reduction in lymphocyte apoptosis may be a beneficial process and future work should explore the non-oxidant mediated functions of lycopene.

Over 60% of the global population are described as sedentary (WHO, 2002). It is possible that these individuals would respond to tomato supplementation in a different manner to the habitually active individuals used in the final study reported in this thesis. Therefore, future work should examine the impact of antioxidant supplementation (including lycopene) in a sedentary population as this group of people may benefit more from a nutritional intervention than individuals who are frequently active. In addition, it is also quite possible that elite athletes involved in extremely demanding training schedules could also benefit from antioxidant intervention. What appears more certain, however, is that because the absolute level of regular physical activity has a direct role in modulating antioxidant defences, investigators should take this into account when designing future experiments that aim to examine the impact of dietary modification.

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APPENDICES

Appendix 1: Medical History Questionnaire

Health Screen Questionnaire

Name: _____

Date: _____

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate.

1. At present, do you have any health problem for which you are:

- | | | |
|--|------------------------------|-----------------------------|
| (a) On medication, prescribed or otherwise | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) Attending your general practitioner | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) On a hospital waiting list | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

2. In the past two years, have you had any illness which requires you to:

- | | | |
|---|------------------------------|-----------------------------|
| (a) Consult your GP | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) Attend a hospital outpatient department | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) Be admitted to hospital | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

3. Do you, or have you ever had, any of the following:

- | | | |
|--|------------------------------|-----------------------------|
| (a) Convulsion/epilepsy | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) Asthma | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) Diabetes | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (d) Any blood disorder | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (e) Head injury | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (f) Digestive problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (g) Heart problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (h) Problems with bones or joints | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (i) Disturbance of balance/co-ordination | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (j) Ear/hearing problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (k) Thyroid problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

(l) Kidney or liver problems

Yes ☐No ☐

(m) Problems with your diet

Yes ☐No ☐

If **YES** to any question, please describe briefly if you wish (e.g. to confirm whether the problem was/is short-lived, insignificant or well-controlled).

.....

Additional questions for **female** participants

(a) Are your periods normal/regular?

Yes ☐No ☐

(b) Are you on 'the pill'?

Yes ☐No ☐

(c) Could you be pregnant?

Yes ☐No ☐

(d) Are you on hormone-replacement therapy?

Yes ☐No ☐

Signature _____

Date _____

BELOW: To be completed on subsequent visits to the laboratory, when these visits are more than one week after the form was first completed.

Please read the form again. If anything has changed since your first visit, please use the space below to provide information:

.....

Appendix 2: Activity Status Questionnaire

How many times per week do you exercise?

Do you exercise individually or with others, please specify?

Eg. 30 min run by myself, passing practice with team mates

Which sport (s) do you partake in on a regular basis i.e. weekly?

In your opinion, what do you view as aerobic exercise?

In your opinion, what do you view as challenging exercise?

How many years have you participated in your main sport?

What level did you achieve?

Recreation

Club

County

District

National

International

Please detail a typical weekly training schedule (table 1) including the categories below. This can be broken up in to daily sessions of exercise for ease. Please specify the duration for each category that applies to you.

Warm up (WU)/ Recovery work (R) (HR < 140 bpm)	eg. 20 min W and 10 R
Low intensity aerobic exercise (LI) (HR 140-160 bpm)	eg. 30 + min
Moderate intensity exercise (MI) (HR 160-175 bpm)	eg. 10-30 min
High intensity exercise (HI) (HR 175+ bpm)	eg. Sprints, max effort
Strength/ Resistance work (S)	
Skill specific work (drills) (SS)	
Other	

Table 1 Typical Exercise Regime

Monday	
Tuesday	
Wednesday	
Thursday	
Friday	
Saturday	
Sunday	

Appendix 3: Foods containing lycopene

These foods contain lycopene. Please avoid all these foods for the whole 8 weeks of the study. If you are unsure about a food please check the ingredients list or contact me.

Apricots
Asparagus
Beef noodle soup
Beef and vegetable soup
Baked beans
BBQ sauce
Branston pickle
Brown sauce
Carrots, raw
Carrot juice
Chilli powder
Chopped tomatoes
Cinnamon, ground
Curry sauces (tomato based)
Dried parsley
Grapefruit (pink and red)
Ketchup
Minestrone condensed soup
Passata
Pasta sauce
Pizza
Pork sausages
Plum tomatoes
Red cabbage
Red peppers
Salad dressing, French
Salad dressing, Italian
Salsa
Seafood sauce
Spaghetti in tomato sauce
Sun dried tomatoes
Sweet & sour sauce
Thousand Island dressing

Tomatoes, red ripe
Tomatoes, plum
Tomato Frito
Tomato juice
Tomato ketchup
Tomato paste, w/o salt
Tomato puree, w/o salt
Tomato sauce, pasta based
Tomato sauce
Tomato condensed soup
Tomato soup
Vegetable juice
Vegetable juice cocktail
Vegetable soup
Watermelon

The meals below contain some of the foods listed, so take care to avoid these in particular:

Spaghetti bolognese
Chilli con carne
Lasagne
Certain curry dishes
Pizzas
Seafood dishes (red)
Enchiladas
Cannelloni

Appendix 4: Cell viability following H₂O₂ treatment

TK6 cells were treated with H₂O₂ in serum free media (SFM) or PBS for 30 min and cell viability was determined immediately following treatment using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The MTT assay is based on reduction of the tetrazolium salt, MTT, by actively growing cells to produce a blue formazan product (Berridge and Tan, 1993). The level of MTT cleavage by viable cells and hence the amount of formazan produced, is directly proportional to the cell number, enabling quantification of viable cells and cytotoxicity (Gerlier and Thomasset, 1986).

Briefly, MTT stock solution was dissolved in SFM (1 ml of 5 mg/ml MTT diluted in 10 ml SFM) and added to cells following the recovery incubation. Samples diluted in MTT were added in triplicate (200 µl/sample) to a 96 well plate, and incubated for 3 h at 37 °C. Following incubation, the MTT solution was aspirated and 100 µl DMSO was added to each well prior to analysis in a microplate reader at 550 nm (MR5000, Dynatech Laboratories, West Sussex, UK).

A paired *t* statistic was performed showing no differences in the cell viability between treatment media (*P* > 0.05) (Figure A.1). As a result, PBS was the treatment media of choice for future experiments.

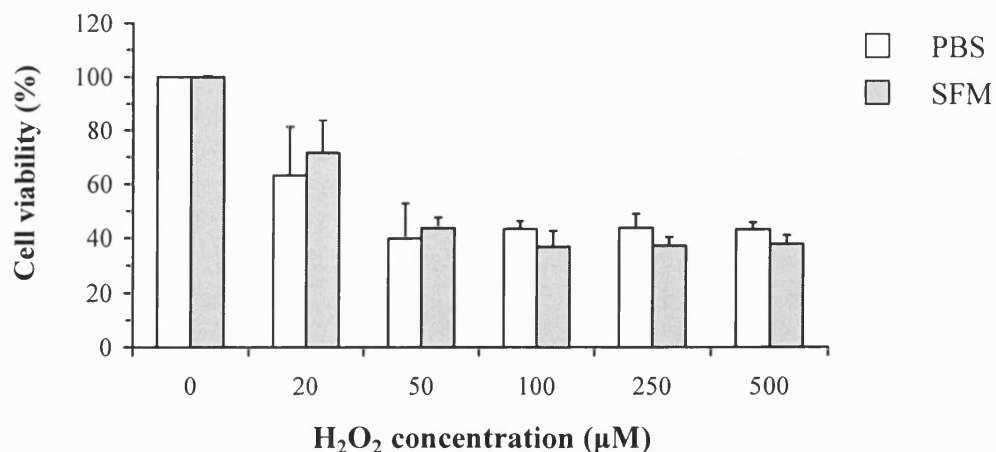
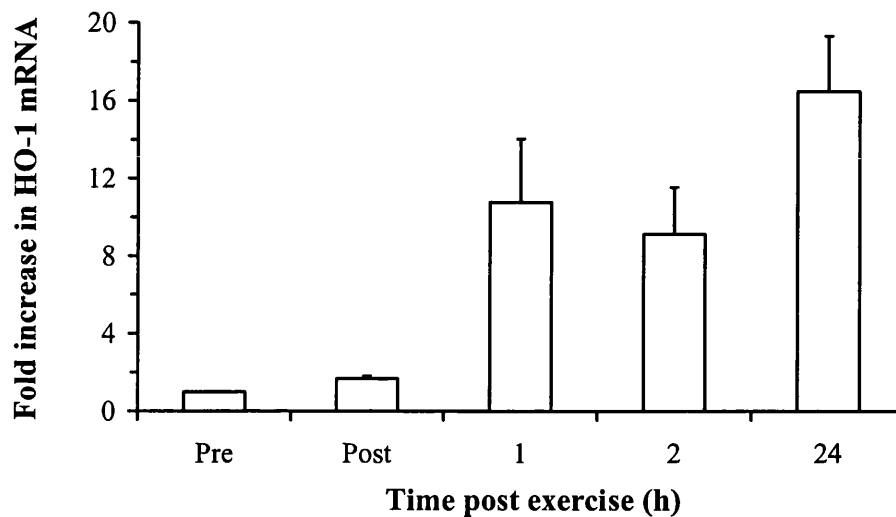


Figure A.1 Cell viability of TK6 cells immediately following 30 min treatment with H₂O₂ in serum free media (SFM) or PBS. Values are mean ± S.D. (n=3).

Appendix 5: Reproducibility of the light cyclers runs

(A)



(B)

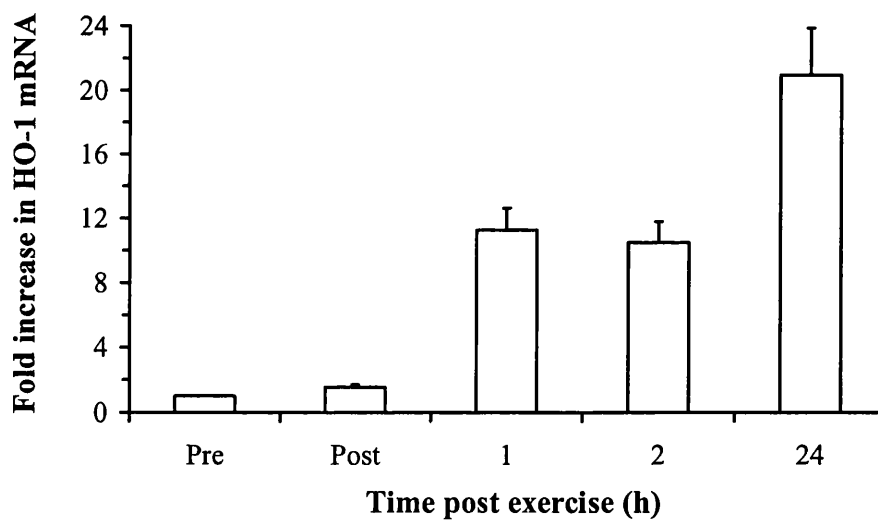


Figure A.2 The fold change in HO-1 mRNA expression following running at 75 min at 70% $\dot{V}O_2$ max demonstrating the reproducibility of the various stages of real-time RT-PCR, with the light cyclers being used for three separate reverse transcriptions of the same RNA (A) and three separate light cyclers runs on one aliquot of cDNA (B). The fold change in HO-1 mRNA was calculated by normalising to GAPDH, and then expressed relative to baseline (Pre). Pre: pre-exercise, Post: 0 h post-exercise. Values are mean \pm S.D. (n=3).

Appendix 6: Preliminary result from the intermittent treadmill protocol

The intermittent exercise protocol was performed by six individuals prior to being used in the main trial (Chapter 5) in order to ensure that the exercise demands and duration were manageable. Subjects 1 to 4 participated in intermittent sports (mainly squash, tennis and rugby) with subjects 5 and 6 participating in endurance activities (triathlon). The $\dot{V}O_2\text{max}$ ($\text{ml.kg}^{-1}\text{min}^{-1}$) are indicated for each subject with their corresponding speeds for the different intensities of the protocol (Table A.1). Subjects 1-3 completed the protocol although subjects 4-6 did not; with subject 6 only managing 20 min. The reasons for this may be due to their level of fitness and efficiency of running which was higher in subjects 4-6 or the fact that they were not used to changing running speeds unlike subjects 1-3.

Table A.1 Speeds for each subject corresponding to the three intensities of the intermittent running protocol and the duration of exercise completed

Subject	$\dot{V}O_2\text{max}$ ($\text{ml.kg}^{-1}\text{min}^{-1}$)	Speed at % $\dot{V}O_2\text{max}$ (km.h^{-1})			Duration (min)
		65%	85%	100%	
1	51.0	9.0	12.3	14.8	60
2	52.4	9.1	11.9	14.0	60
3	64.3	11.1	14.0	16.1	60
4	62.6	11.5	14.9	17.5	44
5	70.7	12.2	15.9	18.7	34
6	73.3	13.0	17.3	20.5	20

Appendix 7: The effect of H₂O₂ concentration on lymphocyte viability

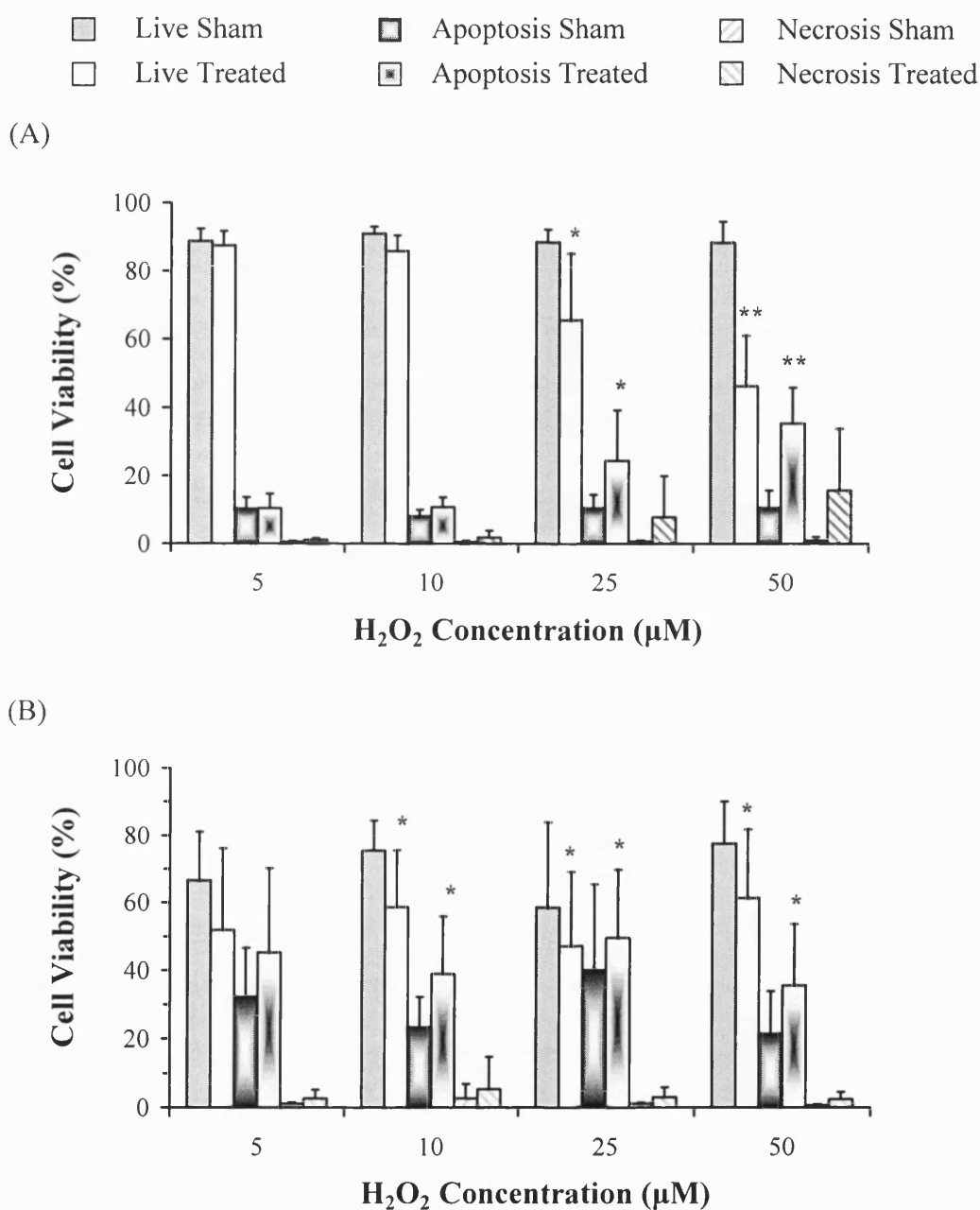
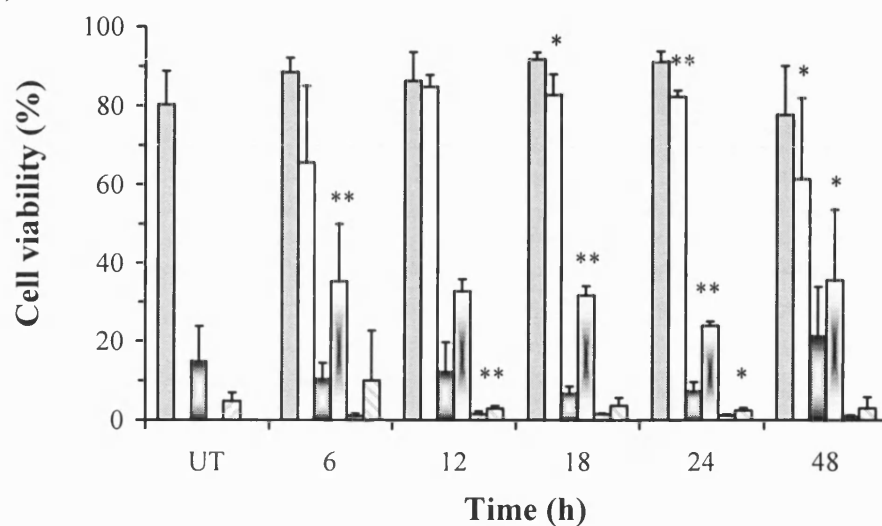


Figure A.3 The effect of H₂O₂ concentration on cell viability 6 h (A) and 48 h (B) following 30 min incubation with H₂O₂. ** denotes a significant difference between the sham and treated samples for a respective concentration ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=5).

Appendix 8: The time course of lymphocyte viability following H₂O₂ treatment

■ Live Sham ■ Apoptosis Sham ▨ Necrosis Sham
 □ Live Treated ■ Apoptosis Treated ▨ Necrosis Treated

(A)



(B)

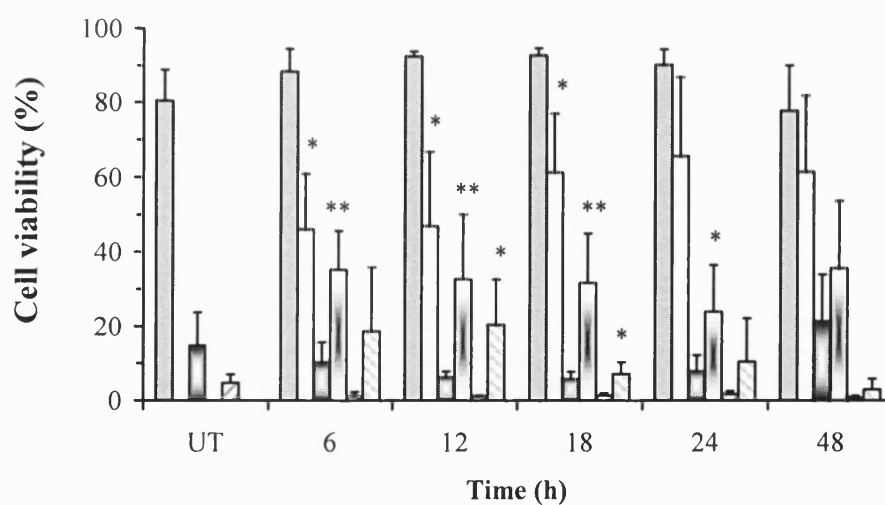
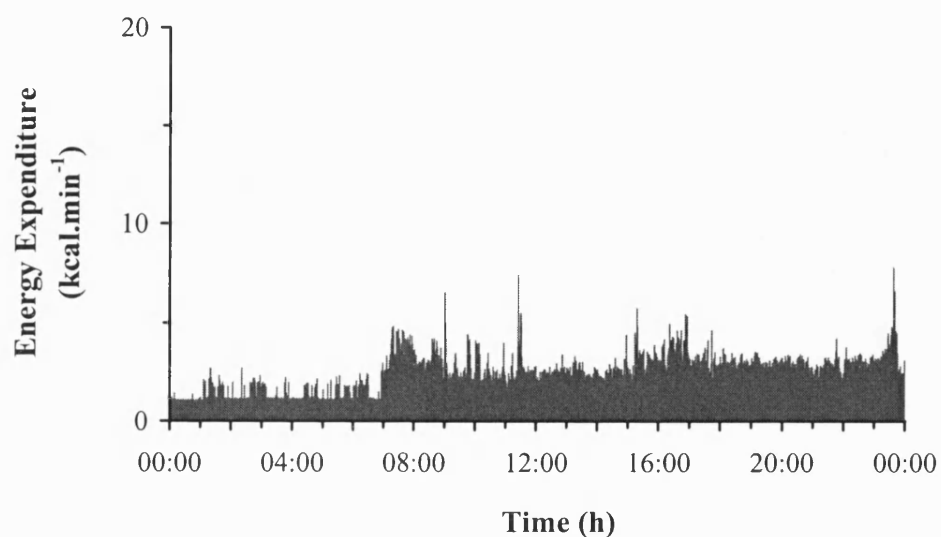


Figure A.4 The effect of time on cell viability following 30 min incubation with 25 μ M H₂O₂ (A) and 50 μ M H₂O₂ (B). ** denotes a significant difference between the sham and treated samples at each respective time point ($P < 0.01$), * ($P < 0.05$). Values are mean \pm S.D. (n=5).

Appendix 9: Acti-heart analysis (Chapter 6)

(A)



(B)

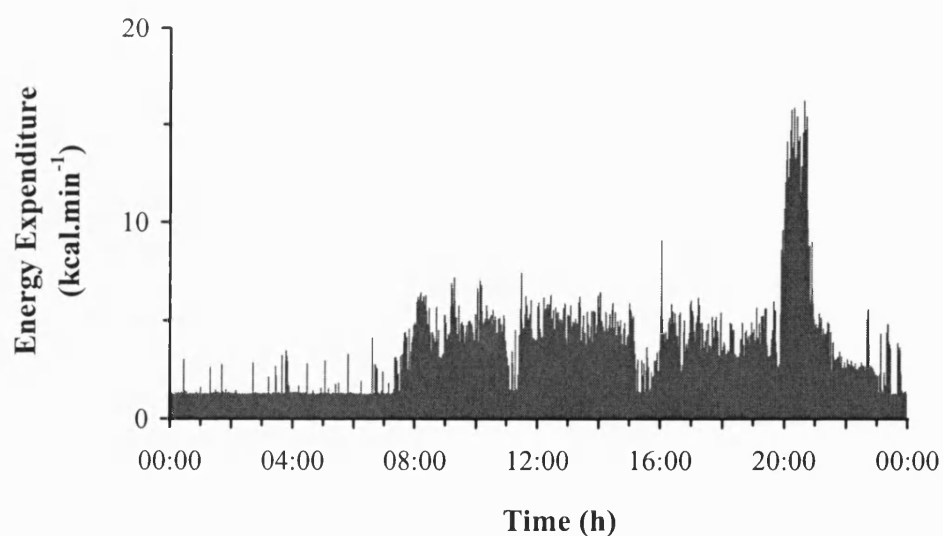


Figure A.5 Energy expenditure analysis from the acti-heart data in the least active individual (A) and the most active individual (B). The energy expenditure (kcal.min⁻¹) represents the daily total energy expenditure (TEE = BMR + AEE).

Appendix 10: Relationships between physical activity, HO-1 and cell death

Table A.2 The relationship between min engaged in levels of physical activity and basal level of HO-1 protein and cell death (A), HO-1 protein response to H₂O₂ treatment (B) and cell death response to H₂O₂ treatment (C)

(A)	BASELINE					NO TOMATO					TOMATO				
	HO-1					HO-1					HO-1				
	Ly	Mon	L	A	N	Ly	Mon	L	A	N	Ly	Mon	L	A	N
Total															
rho	0.310	0.284	-0.092	-0.073	0.189	-0.149	-0.156	-0.229	0.182	0.197	-0.041	0.032	-0.174	0.153	0.103
P value	0.140	0.179	0.668	0.734	0.377	0.498	0.488	0.282	0.395	0.357	0.851	0.881	0.416	0.475	0.630
Moderate															
rho	0.193	0.117	-0.166	0.017	0.252	-0.292	-0.303	-0.209	0.282	-0.174	-0.198	-0.090	-0.104	-0.046	0.213
P value	0.367	0.587	0.439	0.939	0.235	0.176	0.170	0.326	0.181	0.417	0.354	0.675	0.627	0.831	0.318
Vigorous															
rho	0.351	0.413	-0.15	-0.053	-0.038	0.171	0.189	-0.119	-0.026	0.525	0.194	0.218	-0.243	0.272	0.008
P value	0.093	0.045	0.944	0.807	0.859	0.434	0.399	0.579	0.904	0.008	0.364	0.307	0.253	0.199	0.971

Ly: lymphocytes, Mon: monocytes, L: live cells, A: apoptotic cells, N: necrotic cells, rho: Spearman's rank correlation. **Bold text denotes modest relationships (P < 0.1).** (n=24).

Table A.2 (B) The relationship between min engaged in levels of physical activity and HO-1 protein response to H₂O₂ treatment

(B)	NO TOMATO (HO-1)				TOMATO (HO-1)			
	18 h Ly	18 h Mon	48 h Ly	48 h Mon	18 h Ly	18 h Mon	48 h Ly	48 h Mon
Total								
rho	0.132	0.120	0.146	0.053	-0.314	-0.014	0.017	-0.156
<i>P</i> value	0.539	0.575	0.497	0.806	0.135	0.948	0.939	0.467
Moderate								
rho	0.087	-0.057	0.146	-0.033	-0.500	0.091	0.040	-0.067
<i>P</i> value	0.684	0.792	0.497	0.880	0.013	0.671	0.854	0.755
Vigorous								
rho	0.260	0.384	0.148	0.168	0.106	-0.199	0.066	-0.192
<i>P</i> value	0.220	0.064	0.490	0.431	0.623	0.350	0.759	0.368

Ly: lymphocytes, Mon: monocytes, L: live cells, A: apoptotic cells, N: necrotic cells, rho: Spearman's rank correlation. **Bold text denotes modest relationships ($P < 0.1$).** (n=24).

Table A.2 (C) The relationship between min engaged in levels of physical activity and cell death response to H₂O₂ treatment

(C)	NO TOMATO						TOMATO					
	18 h			48 h			18 h			48 h		
	L	A	N	L	A	N	L	A	N	L	A	N
Total												
rho	0.031	0.008	0.039	0.472	-0.449	-0.391	0.003	-0.020	-0.004	0.004	0.251	0.267
P value	0.886	0.971	0.856	0.041	0.054	0.098	0.987	0.926	0.986	0.989	0.300	0.270
Moderate												
rho	0.213	-0.254	-0.020	0.515	-0.487	-0.402	-0.134	-0.089	0.131	-0.022	0.248	0.152
P value	0.319	0.231	0.928	0.024	0.034	0.088	0.532	0.680	0.542	0.929	0.307	0.535
Vigorous												
rho	0.004	0.177	-0.047	0.385	-0.322	-0.402	0.168	0.101	-0.233	0.063	0.119	0.198
P value	0.984	0.408	0.827	0.104	0.179	0.088	0.433	0.637	0.274	0.797	0.629	0.418

Ly: lymphocytes, Mon: monocytes, L: live cells, A: apoptotic cells, N: necrotic cells, rho: Spearman's rank correlation. **Bold text denotes modest relationships (P < 0.1).** (n=24 at 18 h, n=19 at 48 h).